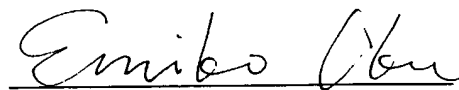


## DECLARATION

I, Emiko Oku of HIROTA & ASSOCIATES, residing at Wakabayashi Bldg., 3F, 8-5, Akasaka 2-chome, Minato-ku, Tokyo 107-0052, Japan, do hereby certify that I am conversant with the English and Japanese languages and am a competent translator thereof, and I further certify that to the best of my knowledge and belief the following is a true and correct translation made by me of the document in the Japanese language filed for a patent application in Japan under No. 2000-219652 on July 19, 2000 in the name of JAPAN SCIENCE AND TECHNOLOGY CORPORATION at Tokyo, Japan, entitled: "Receptor Proteins Specifically Recognizing Bacterial DNA".

Signed this 24th day of October, 2006

A handwritten signature in cursive script, appearing to read "Emiko Oku", written in dark ink.

Emiko Oku

PATENT OFFICE  
JAPANESE GOVERNMENT

This is to certify that the annexed is a true copy of the following  
application as filed with this Office.

Date of Application: July 19, 2000

Application Number: Patent Application No. 2000-219652

Applicant(s): JAPAN SCIENCE AND TECHNOLOGY CORPORATION

Commissioner, Kozo Oikawa (Sealed)  
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【Name of Document】 Specification

【Title of the Invention】 Receptor Proteins Specifically Recognizing Bacterial DNA

【Scope of Claims】

【Claim 1】 DNA encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence.

【Claim 2】 The DNA according to claim 1 wherein a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence is either of the following proteins (a) or (b): (a) a protein comprising the sequence of amino acids shown in Seq. ID No: 2, or (b) a protein comprising a sequence of amino acids wherein one or more of amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No: 2, and having reactivity against bacterial DNA having an unmethylated CpG sequence.

【Claim 3】 The DNA according to claim 1 comprising the sequence of bases shown in Seq. ID No: 1 or its complementary sequence, or part or whole of the sequences.

【Claim 4】 The DNA according to claim 1 which hybridizes with the DNA comprising a gene according to claim 3 under a stringent condition.

【Claim 5】 The DNA according to claim 1 wherein a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence is either of the following proteins (a) or (b): (a) a protein comprising the sequence of amino acids shown in Seq. ID No: 4, or (b) a protein comprising a sequence of amino acids wherein one or more of amino acid are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No: 4, and having reactivity against bacterial DNA having an unmethylated CpG sequence.

【Claim 6】 The DNA according to claim 1 comprising the sequence of bases shown in Seq. ID No: 3 or its complementary sequence, or part or whole of the sequences.

【Claim 7】 The DNA according to claim 1 which hybridizes with the DNA comprising the gene according to claim 6 under a stringent condition.

【Claim 8】 A receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence.

【Claim 9】 The protein according to claim 8 comprising the sequence of amino acids shown in Seq. ID No: 2.

【Claim 10】 The protein according to claim 8 comprising a

sequence of amino acids wherein one or more of amino acids are deleted, substituted or added in the sequence of amino acids shown in Seq. ID No: 2.

【Claim 11】 The protein according to claim 8 comprising the sequence of amino acids shown in Seq. ID No: 4.

【Claim 12】 The protein according to claim 8 comprising a sequence of amino acids wherein one or more of amino acids are deleted, substituted or added in the sequence of amino acids shown in Seq. ID No: 4.

【Claim 13】 A fusion protein comprising the protein according to any one of claims 8 to 12 fused with a marker protein and/or a peptide tag.

【Claim 14】 An antibody specifically bound to the protein according to any one of claims 8 to 12.

【Claim 15】 The antibody according to claim 14 which is a monoclonal antibody.

【Claim 16】 A host cell comprising an expression system expressing the protein according to any one of claims 8 to 12.

【Claim 17】 A non-human animal wherein a gene encoding a receptor protein specifically recognizing bacterial DNA having

an unmethylated CpG sequence is excessively expressed.

【Claim 18】 A non-human animal wherein a gene function encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence is destroyed on a chromosome.

【Claim 19】 The non-human animal according to claim 18 having no reactivity against bacterial DNA having an unmethylated CpG sequence.

【Claim 20】 The non-human animal according to any one of claims 17 to 19 characterized in that a rodent animal is a mouse.

21. A method of preparing a cell expressing a protein having reactivity against bacterial DNA having an unmethylated CpG sequence characterized in that the DNA according to any one of claims 1 to 7 is introduced into a cell wherein a gene function encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence is destroyed on a chromosome.

【Claim 22】 A cell expressing a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence obtained by the method of preparing a cell expressing a receptor protein specifically recognizing bacterial DNA having an



unmethylated CpG sequence according to claim 21.

【Claim 23】 A screening method for an agonist or an antagonist of a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence comprising steps of: invitro culturing a cell expressing a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence in the presence of a target substance, and measuring/evaluating TLR9 activity.

【Claim 24】 A screening method for an agonist or an antagonist of a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence comprising steps of: administrating a target substance to a non-human animal wherein a gene function encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence is destroyed on a chromosome, and measuring/evaluating TLR9 activity of macrophages or spleen cells obtained from the non-human animal.

【Claim 25】 A screening method for an agonist or an antagonist of a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence comprising steps of:

administering a target substance to a non-human animal wherein a gene encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence is excessively expressed, and measuring/evaluating TLR9 activity of macrophages or spleen cells obtained from the non-human animal.

**【Claim 26】** A screening method for an agonist or an antagonist of a protein having reactivity against bacterial DNA having the unmethylated CpG sequence according to either of claims 24 or 25 using a mouse as a non-human animal.

**【Claim 27】** An agonist or an antagonist of a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence obtained by the screening method for an agonist or an antagonist of a receptor protein specifically recognizing bacterial DNA having the unmethylated CpG sequence according to any one of claims 23 to 26.

**【Claim 28】** A pharmaceutical composition comprising whole or part of a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence as an active component.

**【Claim 29】** A pharmaceutical composition comprising the agonist or antagonist according to claim 27 as an active

component.

【Claim 30】 A kit used to diagnose diseases related to the deletion, substitution and/or addition in a sequence of DNA encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence comprising the DNA according to claim 3, which can compare a sequence of DNA encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence in a test body with a sequence of bases in the DNA according to claim 3.

【Detailed Description of the Invention】

【0001】

【Technical Field to Which the Invention Pertains】

The present invention relates to a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence, a gene of the receptor protein and uses of them.

【0002】

【Prior Art】

It is already known that Toll genes are necessary for determining the dorsoventral axis in the embryogeny of *Drosophila* (Cell 52, 269-279, 1988, Annu Rev. Cell Dev. Biol. 12, 393-416, 1996) and for antifungal immune responses in the adult fly (Cell 86, 973-983, 1996).

It has been shown that the Toll is a Type I transmembrane receptor comprising leucine-rich repeat (LRR) in extracellular domains, and its intracellular domains are highly homologous to the intracellular domains of mammalian interleukin-1 receptor (IL-1R) (Nature 351, 355-356, 1991, Annu. Rev. Cell Dev. Biol. 12, 393-416, 1996, J. Leukoc. Biol. 63, 650-657, 1998).

**【0003】**

Recently, mammalian homologs of Toll called Toll-like Receptor (TLR) have been identified, and six members of the family such as TLR2 and TLR4 have been reported (Nature 388, 394-397, 1997, Proc. Natl. Acad. Sci. USA 95, 588-593, 1998, Blood 91, 4020-4027, 1998, Gene 231, 59-65, 1999). It is known that a member of the TLR family mediates MyD88, an adapter protein as IL-1R is, recruits IL-1R binding kinase (IRAK), activates TRAF6, and activates downstream NF- $\kappa$ B (J. Exp. Med. 187, 2097-2101, 1998, Mol. Cell 2, 253-258, 1998, Immunity 11, 115-122, 1999). It is also thought that the role of the TLR family in mammals is related to innate immune recognition as a pattern recognition receptor (PRR) recognizing bacterial common components (Cell 91, 295-298, 1997).

【0004】

It is well known that one of the pathogen-associated molecular patterns (PAMP) recognized by the PRR mentioned above is lipopolysaccharide (LPS), which is a main component of the outer membrane of Gram-negative bacteria (Cell 91, 295-298, 1997), the LPS stimulates a host cell to produce various inflammatory cytokines such as TNF  $\alpha$ , IL-1 or IL-6 in the host cell (Adv. Immunol. 28, 293-450, 1979, Annu. Rev. Immunol. 13, 437-457, 1995), and the LPS captured by LPS-binding protein (LBP) is transferred to CD 14 on the surface of a cell (Science 249, 1431-1433, 1990, Annu. Rev. Immunol. 13, 437-457, 1995). The present inventors generated TLR4 knockout mice and reported that the TLR4 knockout mice lack the ability to respond to LPS, a main component of the outer membrane of the Gram-negative bacteria (J. Immunol. 162, 3749-3752, 1999), and also generated TLR2 knockout mice and reported that macrophages derived from TLR2 knockout mice showed low levels of response to cell wall of Gram-negative bacteria or peptidoglycan, a component of the Gram-negative bacteria (Immunity 11, 443-451, 1999).

【0005】

On the other hand, from the fact that the oligonucleotides comprising bacterial DNA (DNA derived from bacteria) or an unmethylated CpG sequence stimulate immune cells of mice or human (Trends Microbiol. 4, 73-76, 1996, Trends Microbiol. 6, 496-500, 1998), and stimulate a T helper 1 cells (Th1)-like inflammatory response dominated by the release of IL-12 and IFN $\gamma$  (EMBO J. 18, 6973-6982, 1999, J. Immunol. 161, 3042-3049, 1998, Proc. Natl. Acad. Sci. USA 96, 9305-9310, 1999), it is advocated that the oligonucleotides comprising CpG sequence are possibly used as an adjuvant in vaccine strategies including vaccines to cancer, allergy and infectious diseases (Adv. Immunol. 73, 329-368, 1999, Curr. Opin. Immunol. 12, 35-43, 2000, Immunity 11, 123-129, 1999). Although its effects have been expected in the clinical practice in this way, the molecular mechanism by which bacterial DNA comprising an unmethylated CpG sequence activates immune cells is unclear.

**[0006]**

**[An Object to be Attained]**

Although the DNA derived from bacteria comprising an unmethylated CpG motif activates immune cells significantly and

induces response by Th1 as mentioned above, the activities at the molecular level are not well understood. The goal of the present invention is to provide a receptor protein TLR9, a member of TLR family specifically recognizing bacterial DNA comprising an unmethylated CpG sequence, the DNA encoding it, and the artificial animal models useful in examining response of host immune cells to bacterial infectious diseases, which elucidate effects of oligonucleotides comprising an unmethylated CpG sequence of bacterial DNA at the molecular level.

【0007】

【Means to Attain the Object】

As a member of the mammalian TLR family, a pattern recognition receptor recognizing common structures of bacteria, relevant to innate immune recognition, six members (TLR1 to 6) have been publicized until now (Nature 388, 384-397, 1997, Proc. Natl. Acad. Sci. USA, 95, 588-593, 1998, Gene 231, 59-65, 1999), and TLR7 and TLR8, two novel members, are registered in GenBank (Registration No: AF240467 and AF246971). Although full-length cDNA is also found out for TLR9, and is registered in GenBank (Registration No: AF245704), its function has not been known.

【0008】

The present inventors screened the DNA encoding TLR family member receptor proteins specifically recognizing bacterial DNA comprising an unmethylated CpG sequence on BLAST search, screened a number of sequence tagged (EST) clones highly homologous to various TLR already identified, isolated full-length cDNA from mouse macrophage cDNA library by using the fragments as a probe. We also isolated the human cDNA in the same manner. Next, the sequences of bases of the cDNA were examined, and it was confirmed that it is TLR9, in which regions conserved in the TLR family such as LRR and TIR domains are present. We generated TLR9 knockout mice, showed that TLR9 is a receptor protein to the oligonucleotides comprising an unmethylated CpG sequence of bacterial DNA and completed the invention.

【0009】

The present invention relates to DNA encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence (claim 1), the protein according to claim 1 wherein a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence is either of



the following proteins (a) or (b): (a) a protein comprising the sequence of amino acids shown in Seq. ID No: 2, or (b) a protein comprising a sequence of amino acids wherein one or more of amino acids are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No: 2, and having reactivity against bacterial DNA having an unmethylated CpG sequence (claim 2), the DNA according to claim 1 comprising the sequence of bases shown in Seq. ID No: 1 or its complementary sequence, or part or whole of the sequences (claim 3), the DNA according to claim 1 which hybridizes with the DNA comprising a gene according to claim 3 under a stringent condition (claim 4), the protein according to claim 1 wherein a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence is either of the following proteins (a) or (b): (a) a protein comprising the sequence of amino acids shown in Seq. ID No: 4, or (b) a protein comprising a sequence of amino acids wherein one or more of amino acid are deleted, substituted, or added in the sequence of amino acids shown in Seq. ID No: 4, and having reactivity against bacterial DNA having an unmethylated CpG sequence (claim 5), the DNA according to claim 1 comprising the

sequence of bases shown in Seq. ID No: 3 or its complementary sequence, or part or whole of the sequences (claim 6), and the DNA according to claim 1 which hybridizes with the DNA comprising the gene according to claim 6 under a stringent condition (claim 7).

**【0010】**

The present invention also relates to a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence (claim 8), the protein according to claim 8 comprising the sequence of amino acids shown in Seq. ID No: 2 (claim 9), the protein according to claim 8 comprising a sequence of amino acids wherein one or more of amino acids are deleted, substituted or added in the sequence of amino acids shown in Seq. ID No: 2 (claim 10), the protein according to claim 8 comprising the sequence of amino acids shown in Seq. ID No: 4 (claim 11), and the protein according to claim 8 comprising a sequence of amino acids wherein one or more of amino acids are deleted, substituted or added in the sequence of amino acids shown in Seq. ID No: 4 (claim 12).

**【0011】**

The present invention also relates to a fusion protein comprising the protein according to any one of claims 8 to 12 fused with a marker protein and/or a peptide tag (claim 13), an antibody specifically bound to the protein according to any one of claims 8 to 12 (claim 14), the antibody according to claim 14 which is a monoclonal antibody (claim 15), a host cell comprising an expression system expressing the protein according to any one of claims 8 to 12 (claim 16).

**[0012]**

The present invention also relates to a non-human animal wherein a gene encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence is excessively expressed (claim 17), a non-human animal wherein a gene function encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence is destroyed on a chromosome (claim 18), the non-human animal according to claim 18 having no reactivity against bacterial DNA having an unmethylated CpG sequence (claim 19), the non-human animal according to any one of claims 17 to 19 characterized in that a rodent animal is a mouse (claim 20).

【0013】

The present invention also relates to a method of preparing a cell expressing a protein having reactivity against bacterial DNA having an unmethylated CpG sequence characterized in that the DNA according to any one of claims 1 to 7 is introduced into a cell wherein a gene function encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence is destroyed on a chromosome (claim 21), and a cell expressing a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence obtained by the method of preparing a cell expressing a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence according to claim 21 (claim 22).

【0014】

The present invention also relates to screening method for an agonist or an antagonist of a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence comprising steps of: in vitro culturing a cell expressing a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence in the presence of a target substance,

and measuring/evaluating TLR9 activity (claim 23), a screening method for an agonist or an antagonist of a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence comprising steps of: administering a target substance to a non-human animal wherein a gene function encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence is destroyed on a chromosome, and measuring/evaluating TLR9 activity of macrophages or spleen cells obtained from the non-human animal (claim 24), a screening method for an agonist or an antagonist of a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence comprising steps of: administering a target substance to a non-human animal wherein a gene encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence is excessively expressed, and measuring/evaluating TLR9 activity of macrophages or spleen cells obtained from the non-human animal (claim 25), a screening method for an agonist or an antagonist of a protein having reactivity against bacterial DNA having the unmethylated CpG sequence according to either of claims 24 or 25 using a mouse

as a non-human animal (claim 26).

【0015】

The present invention also relates to an agonist or an antagonist of a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence obtained by the screening method for an agonist or an antagonist of a receptor protein specifically recognizing bacterial DNA having the unmethylated CpG sequence according to any one of claims 23 to 26 (claim 27), a pharmaceutical composition comprising whole or part of a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence as an active component (claim 28), a pharmaceutical composition comprising the agonist or antagonist according to claim 27 as an active component (claim 29), a kit used to diagnose diseases related to the deletion, substitution and/or addition in a sequence of DNA encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence comprising the DNA according to claim 3, which can compare a sequence of DNA encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence in a test body with a sequence of bases

in the DNA according to claim 3 (claim 30).

【0016】

【Modes to Carry Out the Invention】

As bacterial DNA comprising an unmethylated CpG sequence in the present invention, any DNA derived from bacteria such as an oligodeoxynucleotide having an unmethylated CpG motif which activates immune cells such as T-cells, B-cells and antigen-presenting cells, and induces immune response can be used such as DNA derived from bacteria including *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella Typhimurium*, *Serratia marcescens*, *Shigella flexneri*, *Vibrio cholerae*, *Salmonella Minnesota*, *Porphyromonas gingivalis*, *Staphylococcus aureus*, *Corynebacterium diphtheriae*, *Nocardia coeliaca*, *Streptococcus pneumoniae*.

【0017】

As a receptor protein specifically recognizing bacterial DNA having the unmethylated CpG motif, there are no particular restrictions as long as the protein can specifically recognize bacterial DNA with an unmethylated CpG sequence, and can be exemplified by human-derived TLR9 shown in Seq. ID No. 2 in the

list of sequence, a protein which comprises a sequence of amino acids wherein one or more of amino acids are deleted, substituted, or added in a sequence of amino acids shown in Seq. ID No: 2, and which specifically recognizes bacterial DNA having the unmethylated CpG sequence, or their recombinant proteins. The receptor protein specifically recognizing bacterial DNA having the unmethylated CpG sequence can be prepared by well known methods based on the information of the DNA sequence and others.

**【0018】**

DNA encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence of the present invention includes DNA encoding human-derived TLR9 shown in Seq. ID No: 2 in the list of sequence such as the one shown in Seq. ID No: 1, DNA comprising a sequence of amino acids wherein one or more of amino acids are deleted, substituted or added in a sequence of amino acids shown in Seq. ID No: 2, and which can specifically recognize bacterial DNA having the unmethylated CpG sequence mentioned above, or DNA hybridized with the DNA under stringent conditions and encoding a protein that can specifically recognize bacterial DNA having the unmethylated



CpG sequence mentioned above. These can be prepared by well known methods based on the information of DNA sequence such as mouse RAW264.7 cDNA library or 129/SvJ mouse gene library for mouse-derived TLR9.

**【0019】**

Further, it is possible to obtain DNA encoding a receptor protein specifically recognizing bacterial DNA having an immune-inducing unmethylated CpG sequence which has the same effect as TLR9, a receptor protein, by hybridizing mouse-derived DNA library with part or whole of a sequence of bases shown in Seq. ID No: 1 or its complementary sequence under stringent conditions to isolate the DNA hybridized with the probe. Conditions on hybridization to obtain the DNA can, for example, be hybridization at 42°C and wash treatment at 42°C with a buffer containing 1%  $\times$  SSC and 0.1% of SDS, and more preferably be hybridization at 65°C and wash treatment at 65°C with a buffer containing 0.1  $\times$  SSC and 0.1% of SDS. Furthermore, beside the temperature conditions mentioned above, there are various factors effecting the stringency of hybridization, and it is possible for a person skilled in the art to realize the stringency

equivalent to the stringency of hybridization illustrated above.

**【0020】**

A fusion protein in the present invention can be the one obtained by combining a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence derived from mouse, human, and others with a marker protein and/or a peptide tag. A marker protein can be any marker protein previously well known, and can be exemplified by alkaline phosphatase, Fc region of an antibody, HRP, GFP and others. As a peptide tag in the present invention, it can be concretely exemplified by previously well-known peptide tags such as Myc tag, His tag, FLAG tag, GST tag. The fusion protein can be produced by a normal method, and is useful in purifying a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence by using affinity of Ni-NTA and His tag, detecting a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence, measuring of the amount of antibodies against a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence and as a research reagent in other relevant fields.

【0021】

As an antibody specifically bound to a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence in the present invention, it can be concretely exemplified by immune-specific antibodies such as a monoclonal antibody, a polyclonal antibody, a chimeric antibody, a single-chain antibody, a humanized antibody. These antibodies can be produced by a normal method by using a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence mentioned above as an antigen, and a monoclonal antibody is preferable in its specificity among them. The antibody specifically bound to a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence such as a monoclonal antibody and others is useful, for example, in diagnosing diseases caused by the mutation or deletion of TLR9 or elucidating the molecular mechanism controlling TLR9.

【0022】

An antibody against a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence can be produced by administering a fragment containing a

receptor protein or an epitope specifically recognizing bacterial DNA having the unmethylated CpG sequence in animals (preferably, non-human), or a cell expressing the protein on the surface of its membrane by a conventional protocol, and any method can be used such as hybridoma method (Nature 256, 495-497, 1975), trioma method, human B cell hybridoma method (Immunology Today 4, 72, 1983), and EBV-hybridoma method (MONOCLONAL ANTIBODIES AND CANCER THERAPY, 77-96, Alan R. Liss, Inc., 1985), which are used for preparing monoclonal antibodies and brings an antibody produced by the cultured successive cell lines. The following explains a method of producing a monoclonal antibody specifically bound to mouse-driven TLR9, that is, an mTLR9 monoclonal antibody, with mouse-driven TLR9 as an example of a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence.

**【0023】**

The mTLR9 monoclonal antibody can be produced by a normal method of culturing hybridoma producing mTLR9 monoclonal antibody in vivo or in vitro. For example, in an in vivo systems they can be obtained by culturing in the visceral cavity of rodents,

preferably of mice or rats, and in an in vitro system they can be obtained by culturing in a medium for culturing animal cells. A medium used for culturing hybridoma in an in vitro system can be exemplified by cell culture media such as RPMI1640 or MEN and others comprising antibiotics such as streptomycin or penicillin.

**【0024】**

The hybridoma producing mTLR9 monoclonal antibody can be produced by immunizing BALB/c mouse with TLR9, a receptor protein obtained from mouse and others, fusing a spleen cell from an immunized mouse and a mouse NS-1 cell (ATCC TIB-18) by a normal method, and screening them by immunofluorescence staining patterns. A method of separating/isolating the monoclonal antibody can be any one as long as it is a method usually used for purifying proteins, and liquid chromatography such as affinity chromatography and others can be a concrete example.

**【0025】**

It is also possible to apply the method of a single-chain antibody (US Patent No. 4946778) to produce single-chain antibodies against receptor proteins specifically recognizing

bacterial DNA having the above-mentioned unmethylated CpG sequence of the present invention. Further, it is possible to use transgenic mice or other mammals and the like to express humanized antibodies, isolate/identify the clones expressing a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence by using the antibodies, and purify the polypeptides by affinity chromatography. The antibodies against receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence are useful in elucidating the molecular mechanism of a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence.

【0026】

It is also possible to carry out a functional analysis of a receptor protein specifically recognizing bacterial DNA having the unmethylated CpG sequence by using fusion proteins obtained by fusing proteins labeled with fluorescent substances such as FITC (fluorescein isothiocyanate) or tetramethylrhodamine isocyanate, fusion proteins labeled with radio isotopes such as  $^{125}\text{I}$ ,  $^{32}\text{P}$ ,  $^{35}\text{S}$  or  $^3\text{H}$ , enzymes such as Alkaline phosphatase, peroxidase,  $\beta$  - Galactosidase or Phycoerythrin, or fluorescent

proteins such as Green Fluorescent Protein (GFP). A method of immunoassay can be exemplified by RIA, ELISA, fluorescence antibody method, plaque forming cell assay, spot method, hemagglutination reaction method, Ouchterlony Method, and others.

【0027】

The present invention relates to a host cell comprising an expressing system that can express a receptor protein specifically recognizing bacterial DNA having the unmethylated CpG sequence. Introduction of a gene encoding a receptor protein specifically recognizing bacterial DNA having the unmethylated CpG sequence into a host cell can be carried out by the methods described in a number of standard laboratory manuals such as in Davis et al. (BASIC METHODS IN MOLECULAR BIOLOGY, 1986) and Sambrook et al. (MOLECULAR CLONING: A LABORATORY MANUAL, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N. Y., 1989), such as calcium phosphate transfection, DEAE-dextran-mediated transfection, transvection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic

introduction, infection and others. A host cell can be exemplified by bacterial prokaryotes such as *Escherichia coli*, *Streptomyces*, *Bacillus subtilis*, *Streptococcus*, *Staphylococcus* and others, fungal cells such as yeast and *Aspergillus*, insect cells such as *Drosophila* S2 or *Spodoptera* Sf9 and others, and animal and plant cells such as L cell, CHO cell, COS cell, Hela cell, C127 cell, BALB/c3T3 cell (including mutant strains lacking dihydrofolate reductase, thymidine kinase or others), BHK 21 cell, HEK293 cell, Bowes Melanoma cell, oocytes, and others.

**[0028]**

Further, the expression system can be any one as long as it is a system that can express a receptor protein specifically recognizing bacterial DNA having the unmethylated CpG sequence in a host cell, and can be exemplified by expression systems derived from chromosome, episome and virus, such as vectors derived from bacterial plasmid, yeast plasmid, papovavirus such as SV40, vaccinia virus, adenovirus, fowl poxvirus, pseudorabies virus, or vectors derived from retrovirus, vectors derived from bacteriophage or transposon or their combinations, which can



be exemplified by plasmids such as cosmid and phagemid, which are derived from genetic factors of plasmids and bacteriophage. These expressing systems may comprise a control sequence that not only causes expression but also regulates expression.

【0029】

A receptor protein specifically recognizing a host cell comprising the expressing system or a cell membrane of the cell, bacterial DNA comprising an unmethylated CpG sequence obtained by culturing, and the cell can be used for the screening methods of the present invention as mentioned below. For example, a method described in F. Pietri-Rouxel et al. (Eur. J. Biochem., 247, 1174-1179, 1997) can be used as a method for obtaining cell membrane, and well known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion- or cation-exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxyapatite chromatography and lectin chromatography, preferably high-performance liquid chromatography can be used to collect a receptor protein specifically recognizing bacterial DNA having the unmethylated

CpG sequence from the cell culture and to purify it. Specifically, it is possible to obtain a receptor protein specifically recognizing the bacterial DNA having an unmethylated CpG sequence by using a column to which a receptor protein antibody specifically recognizing bacterial DNA having the anti-unmethylated CpG sequence of anti-TLR9 monoclonal antibodies and others is bound, or in case an ordinary peptide tag is bound to a receptor protein such as TLR9 etc. specifically recognizing a column to which a substance having an affinity with a peptide tag is bound for affinity chromatography.

**【0030】**

A non-human animal excessively expressing a gene encoding a receptor protein specifically recognizing bacterial DNA having the unmethylated CpG sequence mentioned above in the present invention can be a non-human animal producing a large amount of receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence compared with wild-type non-human animals. Further, a non-human animal whose gene function encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence is deleted

on the chromosome is a non-human animal wherein part or whole of genes encoding receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence on the a chromosome are inactivated by genetic mutations such as damaged, deleted, substituted, and others, and which lost a function of expressing a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence. Although the non-human animal used in the present invention can be exemplified by a non-human animal including rodents such as rabbits, mice, rats and others, it is not restricted to the animals.

**【0031】**

Further, refractory against bacterial DNA having an unmethylated CpG sequence in the present invention means that the reactivity against stimuli by bacterial DNA shown by an organism, or a cell, a tissue or an organ constituting the organism is declined or almost totally lost. Therefore, a non-human animal with refractory against bacterial DNA having an unmethylated CpG sequence in the present invention is a non-human animal such as mice, rats, or rabbits, wherein the an organism's

reactivity against bacterial DNA, or a cell, a tissue or an organ constituting the organism is declined or almost totally lost. Further, stimuli by bacterial DNA can be exemplified by an in vivo stimulus caused by administering bacterial DNA to an organism, or an in vitro stimulus caused by contacting cells separated from an organism with bacterial DNA. Concretely, a non-human animal such as TLR9 knockout mice wherein TLR9 gene functions are destroyed on the chromosome can be an example.

**【0032】**

A homozygote non-human animals born following Mendel's Law includes mice deficient of or excessively expressing receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence and their wild-type littermates, and it is preferable to use wild-type non-human animals, that is, the same kind of animal as a non-human animal wherein gene functions encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence are destroyed or are excessive, more preferably their littermate animals, for example, during the screening of the present invention described below because accurate comparative experiments can be carried

out at the level of individuals by using the homozygote non-human animals with its receptor proteins destroyed or the one with receptor proteins expressing excessively or the wild-type non-human animals born from the same mother at the same time. In the following, a method of producing non-human animals wherein gene functions encoding a receptor protein specifically recognizing bacterial DNA having the unmethylated CpG sequence are destroyed or excessively expressed on the chromosome is explained using knockout mice or transgenic mice whose receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence as an example.

**【0033】**

For example, as for a mouse wherein gene functions encoding receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence are destroyed on a chromosome such as TLR9, that is, a knockout mouse lacking receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence, gene fragments obtained from mouse gene library by a method of PCR or the like are used to screen genes encoding receptor proteins specifically recognizing bacterial DNA having

the unmethylated CpG sequence, subclone a gene encoding a receptor protein specifically recognizing bacterial DNA having the screened unmethylated CpG sequence with viral vectors and others, and specified by DNA sequencing. Whole or part of the gene in the clone encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence is substituted with pMC1 neo gene cassette and others, and a targeting vector is produced by introducing diphtheria toxin A fragments (DT-A) genes or herpes simplex virus thymidine kinase (HSV-tk) genes and others on 3'-end side.

**【0034】**

The produced targeting vector is linearized, introduced into ES cells by electroporation method and others, homologous recombination is performed, and ES cells which has caused homologous recombination by antibiotics such as G418 or gancyclovir (GANC) and others are selected from the homologous recombinants. It is preferable to confirm by Southern blot technique that the selected ES cells are targeted recombinants. The clones of the confirmed ES cells are introduced to mouse blastocysts by microinjection, and the blastocysts are returned

to recipient mice, and chimera mice were produced. The chimera mouse was intercrossed with a wild-type mouse to produce a heterozygote mouse, and the heterozygote mice are intercrossed to produce a knockout mouse lacking a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence in the present invention. Further, a method of confirming whether knockout mice lacking a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence is obtained, for example, may be examined by Northern blot technique, which isolates RNA from the mouse obtained by the method mentioned above, or the expression in the mice may be examined by Western blot technique.

【0035】

The fact that the produced TLR9 knockout mouse is refractory against bacterial DNA having an unmethylated CpG sequence can be confirmed by measuring the levels of the production of TNF- $\alpha$ , IL-6, IL-12, IFN- $\gamma$  and others in the cells whose CpG ODN was contacted in vivo or in vitro with immune cells such as macrophages, mononuclear cells, dendritic cells from TLR9 knockout mice, the proliferation of response of spleen B cells,

the expression of antibodies such as CD40, CD80, CD86, MHC class II on the surface of spleen B cells, and the activation of molecules on the signal transduction pathway of NF- $\kappa$ B, JNK, IRAK and others. The knockout mice lacking TLR9 in the present invention can be used to elucidate functional mechanisms of bacterial DNA and others having an unmethylated CpG sequence and to developing vaccine against bacterial infections.

【0036】

Transgenic mice lacking receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence can be generated by constructing introduced genes by fusing chicken  $\beta$  actin, mouse neurofilament, promoters such as SV40, and rabbit  $\beta$ -globin, polyA such as SV40 or intron with cDNA encoding receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence such as TLR9, microinjecting the introduced genes to pronucleus of mouse fertilized eggs, transplanting the obtained cells to an oviduct of recipient mice after culturing them, then breeding the transplanted animals, and selecting child mice having the cDNA from born child mice. Further, selection of the child mice having



cDNA can be performed by dot hybridization wherein crude cDNA was extracted from mouse tails and others, and genes encoding receptor proteins specifically recognizing bacterial DNA having an introduced unmethylated CpG sequence is used as a probe, or PCR method using specific primers and others.

【0037】

Further, the use of whole or part of DNA encoding receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence in the present invention enables us to prepare cells effective for genetic treatments for diseases caused by the deletion or abnormality of receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence. Methods of preparing the cells in the present invention can be exemplified by a method wherein part or whole of the DNA in the present invention is introduced into cells lacking gene functions encoding receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence on the chromosome by transfection and others, and thus obtaining a cell expressing receptor proteins specifically recognizing bacterial DNA having the unmethylated CpG sequence. It is

preferable to use a cell in which the DNA and others is integrated onto the chromosome and shows TLR9 activity in a stable manner, particularly as a cell expressing receptor proteins specifically recognizing bacterial DNA having the unmethylated CpG sequence.

【0038】

Furthermore, the use of DNA encoding receptor proteins specifically recognizing bacterial DNA having the unmethylated CpG sequence, antibodies against receptor proteins specifically recognizing bacterial DNA having a fused unmethylated CpG sequence comprising a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence bound to a marker protein and/or a peptide tag, a host cell comprising an expression system which can express a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence, non-human animals excessively expressing genes encoding receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence, non-human animals lacking gene functions encoding receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence on a chromosome, cells expressing receptor proteins specifically

recognizing bacterial DNA having an unmethylated CpG sequence enables us to screen agonists or antagonists of the receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence in the present invention, or suppressing or promoting substances reactive to bacterial DNA having an unmethylated CpG sequence. What is obtained by the screening may be suppressing or promoting substances against bacterial infected diseases, suppressing agents, preventing agents or remedies against allergic diseases or cancers, agents suppressing or promoting side effects in genetic therapy or the like, or substances useful for diagnosing/treating diseases or the like caused by the deletion or abnormality of TLR9 activity.

【0039】

Although the TLR activities can concretely be exemplified by a function of reacting specifically to bacterial DNA having an unmethylated CpG sequence and transmitting signals into cells, and a signal transduction function is a function of producing cytokines such as  $\text{TNF-}\alpha$ , IL-6, IL-12,  $\text{IFN-}\gamma$  or the like, a function of producing nitrous acid ion, a function of proliferating cells, a function of expressing antibodies such

as CD40, CD80, CD86, MHC class II and others on the surface of cells, and a function of activating molecules in signal transduction pathway of TLR9 such as NF- $\kappa$ B, JNK, IRAK and others, it is not limited to these functions.

#### 【0040】

A screening method of agonists or antagonists of receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence in the present invention can concretely be exemplified by a method of performing in vitro culture of immune cells such as macrophages, spleen cells or dendritic cells, cells expressing a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence, cells expressing a protein having reactivity against bacterial DNA having an unmethylated CpG sequence in a cell expressing a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence under the presence of target substance, and measuring/evaluating TLR9 activities, or a method of administering target substance to wild-type non-human animals, non-human animals lacking a gene function of encoding receptor proteins specifically recognizing bacterial DNA an

unmethylated CpG sequence, or non-human animals excessively expressing genes encoding receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence, and measuring/evaluating TLR 9 activities of immune cells such as macrophages, spleen cells or dendritic cells derived from these non-human animals.

**【0041】**

Further, in evaluating and measuring the levels of macrophage activities or spleen cell activities, it is preferable to evaluate and compare them with the measurement values obtained from wild-type non-human animals, especially wild-type non-human animals born from the same parent to remove variances arising from individual differences. The same also applies to screening of suppressing or promoting substances reactive to bacterial DNA having an unmethylated CpG sequence shown below.

**【0042】**

Screening methods for suppressing or promoting substances reactive to bacterial DNA having an unmethylated CpG sequence can concretely be exemplified by a method comprising the steps of carrying out in vitro incubation of proteins or cell membranes

expressing the proteins having a reactivity against bacterial DNA having an unmethylated CpG sequence under the presence of target substances and bacterial DNA having an unmethylated CpG sequence, measuring/evaluating the reactivity of the protein, or a method comprising the steps of first making macrophages or spleen cells obtained from non-human animals whose gene functions encoding proteins having reactivity against bacterial DNA having an unmethylated CpG sequence are destroyed on a chromosome contact in vitro with target substances, then culturing the macrophages or spleen cells in the presence of bacterial DNA having an unmethylated CpG sequence, and measuring/evaluating the levels of macrophage activities shown by the macrophages or the levels of spleen cell activities shown by the spleen cells, a method comprising the steps of making macrophages or spleen cells obtained from non-human animals whose gene functions encoding proteins having reactivity against bacterial DNA having an unmethylated CpG sequence contact in vitro with bacterial DNA having an unmethylated CpG sequence, then culturing the macrophages or spleen cells in the presence of target substances, and measuring/evaluating the levels of

macrophage activities shown by the macrophages or the levels of spleen cell activities shown by the spleen cells, and a method of comprising the steps of first administering target substances to non-human animals whose gene functions encoding proteins having reactivity against bacterial DNA having an unmethylated CpG sequence on a chromosome first, then culturing the macrophages or spleen cells obtained from the non-human animals in the presence of bacterial DNA having an unmethylated CpG sequence, and measuring/evaluating the levels of macrophage activities shown by the macrophages or the levels of spleen cell activities shown by the spleen cells, a method comprising the steps of first administering target substances to non-human animals whose gene functions encoding proteins having reactivity against bacterial DNA having an unmethylated CpG sequence is destroyed on a chromosome, then infecting the non-human animals by bacteria, and measuring/evaluating the levels of macrophage activities shown by macrophages or the levels of spleen cell activities shown by the spleen cells obtained from non-human animals, a method of the steps of first administering target substance to non-human animals whose gene functions encoding

proteins having reactivity against bacterial DNA having an unmethylated CpG sequence on a chromosome, and measuring/evaluating the levels of macrophage activities shown by macrophages or the levels of spleen cell activities shown by spleen cells obtained from the non-human animals, a method comprising the steps of first infecting with bacteria non-human animals whose gene functions encoding proteins having reactivity against bacterial DNA having an unmethylated CpG sequence are destroyed on a chromosome, then culturing macrophages or spleen cells obtained from the non-human animals in the presence of target substances, and measuring/evaluating the levels of macrophage activities shown by macrophages or the levels of spleen cell activities shown by spleen cells obtained from the non-human animals, a method comprising the steps of administering target substances to non-human animals whose gene functions are encoding proteins having reactivity against bacterial DNA having an unmethylated CpG sequence are destroyed, infecting the non-human animals by bacteria, and measuring/evaluating the levels of macrophage activities or spleen cell activities in the non-human animals, and a method



comprising the steps of infecting non-human animals whose gene functions encoding proteins having reactivity against bacterial DNA having an unmethylated CpG sequence are destroyed on a chromosome first, then administering the target substances to the non-human animals, and measuring/evaluating the levels of macrophage activities or spleen cell activities in the non-human animals. Although as bacterial DNA having an unmethylated CpG sequence used in the screening methods, it is preferable to use CpG ODN (TCC-ATG-ACG-TTC-CTG-ATG-CT: Seq. ID No: 5), it is not limited to this.

**【0043】**

The present invention also relates to a kit used to diagnose diseases relating to the activity or expression of receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence by comparing a sequence of DNA encoding receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence in a test body with a sequence of DNA encoding receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence in the present invention. The detection of mutated DNA encoding receptor

proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence can be carried out by detecting genetically mutated individuals at the level of DNA, and is effective for diagnosing diseases caused by hypotypic expression, hypertypic expression or mutated expression of receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence. Although a test body used in the detection can concretely be exemplified by genomic DNA of cells from subjects obtainable by biopsy from blood, urine, saliva, tissue and others, RNA, or cDNA, it is not limited to these. In using the test body, it is possible to use the ones amplified by PCR and others. The deficiency or insertional mutation in sequences of bases can be detected by the changes of amplified products in size compared with normal genes, and point mutation can be identified by hybridizing the amplified DNA with the gene encoding receptor proteins specifically recognizing bacterial DNA having labeled unmethylated CpG sequence. It is possible to diagnose or conclude diseases relevant to activity or expression of receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence by detecting mutation of a gene

encoding receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence.

**【0044】**

The present invention also relates to a probe diagnosing a disease related to activities or expressions of a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence comprising whole or part of antisense chain of DNA or RNA encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence, and a kit used to diagnose diseases relating to activities or expressions of a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence comprising an antibody specifically bound to a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence of the probe and/or in the present invention. A probe used for the diagnosis is whole or part of an antisense chain of DNA (cDNA) or RNA (cRNA) encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence, and there is no limitations on the probe as long as it is long enough (at least 20 bases or more) to establish as a probe. In

order to make an antibody specifically bound to a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence of the probe and/or in the present invention an active component of a medicine diagnosing diseases such as bacterial infection and others, it is preferable to dissolve it into appropriate buffers or sterilized water in which a probe is not decomposed. Further, it is possible to use the clinical test pharmaceuticals to diagnose a patient's symptoms such as bacterial infection diseases and others in the ways such as immunofluorescence (Dev. Biol. 170, 207-222, 1995, J. Neurobiol. 29, 1-17, 1996), In situ hybridization (J. Neurobiol. 29, 1-17, 1996), or in situ PCR or others.

**【0045】**

A pharmaceutical composition of the present invention can be any one as long as it comprises whole or part of the receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence of TLR9 or others, or comprises an agonist or an antagonist of the receptor protein. Concretely, vaccines against bacterial infectious diseases, vaccines against cancers, treating medicine for patients having allergies

such as bronchial asthma, reversal agents, suppressing agents, inhibiting agents and others for side effects by the existence of a CpG motif inhibiting genetic treatments or treatments using antisenseoligonucleotides can be exemplified.

**【0046】**

As mentioned above, a kit testing diagnoses relevant to the deletion, substitution and/or addition of DNA sequence encoding a receptor proteins specifically recognizing bacterial DNA having an unmethylated CpG sequence of the present invention can be any one as long as it comprises DNA encoding TLR9, and comparing a sequence of bases of DNA encoding the TLR9 with a sequence of bases of DNA encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence in a tested body enables us to diagnose diseases related to deletion, substitution and/or addition of DNA sequence encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence such as cancer, allergy, infectious diseases and others.

**【0047】**

**【Examples】**

In the following, the present invention will further be explained with concrete examples. However, the technical scope of the present invention is not limited in any way by the following examples.

#### Example 1 (Cloning of TLR9)

As a result of a GenBank search using the information of DNA sequence of human TLR4, a mouse EST having a significant homology (Registration No. AA273731; mouse) was found. Using PCR amplified mouse EST as a probe, mouse RAW 264.7 cDNA library was screened and a full length cDNA clone shown in Seq. ID No. 3 comprising the complete TLR9 open reading frame was isolated. Performing a GenBank search based on the information of DNA sequence of the mouse TLR9, a human genomic sequence having a high level of homology was found. Based on the human genomic sequence, cDNA ends were amplified to isolate cDNA of the full length human TLR9 having a sequence of bases in Seq. ID No. 1 from U937 cells (J. Immunol. 163, 5039-5048, 1999).

**【0048】**

#### Example 2 (Production of TLR knockout mice)

The TLR9 genomic DNA was isolated from 129/SvJ mouse genomic

library (Stratagene), subcloned in pBluescript II SK(+) vectors (Stratagene), and characterized by restriction enzyme mapping and DNA sequencing analysis. The targeting vector was constructed by replacing a 1.0 kb fragment encoding part of LRR (leucine-rich repeat) region with a neomycin-resistance gene cassette (pMC1-neo; Stratagene), and a herpes simplex virus thymidine kinase (HSV-TK) was inserted for negative selection (Fig. 1). The targeting vector was linearized, and was electroporated into embryonic stem cells (ES cells) of E14-1, then 292 pieces of clones showing G418 and gancyclovir resistance were selected, and 14 pieces of clones were screened by PCR and Southern blotting.

【0049】

Chimeric mice were produced by microinjecting 3 pieces of targeted ES clones comprising mutated TLR9 allele into C57BL/6 mouse blastocysts. The male chimeric mice were intercrossed with C57BL/6 female mice to produce a heterozygote F1 mouse, and a homozygote mice (TLR9 knockout mouse:  $TLR9^{-/-}$ ) was obtained by intercrossing heterozygote F1 mouse (Fig. 2). To confirm that the obtained mouse was homozygote, various genomic DNA

extracted from a mouse tail was digested by ScaI to perform Southern blotting using the probe shown in Fig. 1. The TLR9 knockout mice (TLR9<sup>-/-</sup>) of the present invention were produced following Mendel's law, and had not shown remarkable abnormality for 12 weeks.

【0050】

To confirm that the inactivation of TLR9 arises by mutation, total RNA (10  $\mu$ g) extracted from spleen cells from wild-type mice (+/+) and TLR9 knockout mice (-/-) was electrophoresed, and transferred to nylon membranes, Northern blotting was performed with the use of cDNA specific to TLR9 c-terminal fragments or N-terminal fragments labeled with [<sup>32</sup>P], or  $\beta$ -actin (Fig. 3). The result shows that N-terminal fragments of TLR9 mRNA were not detected from the spleen cells of TLR9 knockout mice. Further, with a C-terminal fragment as a probe, almost the same size of Tlr9 transcripts derived from mutated mice as the ones from wild-type mice were detected. However, the amount of the production was small. Then, RT-PCR was performed using mRNA of spleen cells obtained from mutated mice to sequence the obtained products. The result shows that the Tlr9 gene



transcript comprises neo gene, and stop codons appear in a N-terminal domain of TLR9 by inserting the neo, and functional TLR9 proteins does not appear in mutated mice (Fig. 4). Further, as a result of examining lymph cells from TLR9 by flowcytometry knockout mice, no abnormal compositions were found.

**【0051】**

**Example 3 (Preparation of peritoneal macrophages)**

2ml of 4% thioglycolic acid medium (DIFCO) was injected to each peritoneum of wild-type mice and TLR9 knockout mice (TLR9<sup>-/-</sup>), peritoneal exudation cells were isolated from peritonea from each mouse after 3 days, the cells were cultured in RPMI1640 medium to which 10% of fetal bovine serum (GIBCO) was added at 37°C for 2 hours, and remove the unattached cells by washing with ice-chilled Hank's buffered salt solution (HBSS; GIBCO), and the attached cells were used as peritoneal macrophages in the following experiments.

**【0052】**

**Experiment 4 (Response to bacterial DNA having an unmethylated CpG sequence in TLR9 knockout mice)**

It has recently been shown that the response of CpG ODN

(oligodeoxynucleotide) is dependent on MyD88, an adapter protein in a signaling transduction pathway mediating TLR. Although the MyD88 knockout mice do not show response to CpG ODN, TLR2 knockout mice or TLR4 knockout mice show normal response to it. This shows that CpG ODN recognizes TLRs other than TLR2 and TLR4, and then the response of a TLR9 knockout mouse against CpG ODN was examined. First, the amount of producing inflammatory cytokines in peritoneal macrophages were measured in the following way.

**【0053】**

The macrophages prepared in Example 3 are co-cultured with various concentrations of CpG ODN shown in Fig. 5 (0.1 or 1.0  $\mu$ M; TIB MOLBIOL; TCC-ATG-ACG-TTC-CTG-ATG-CT), PGN (10  $\mu$ g/ml; Sigma and Fluka; derived from *Staphylococcus aureus*), LPS (1.0  $\mu$ g/ml; Sigma; derived from *Salmonella minnesota* Re-595) in the presence or absence of  $\text{INF}\gamma$  (30 unit/ml). The concentrations of  $\text{TNF}\alpha$ , IL-6 and IL-12 p40 in the supernatants after culturing were measured by ELISA, and the results are shown in Fig. 5. The results show that the macrophages from wild-type mice (Wild-type) produce  $\text{TNF}\alpha$ , IL-6 and IL-12 in response to CpG

ODN, and further stimulation by  $\text{IFN}\gamma$  and CpG ODN increases the amount of producing  $\text{TNF}\alpha$ , IL-6 and IL-12. However, the macrophages derived from TLR9 knockout mice ( $\text{TLR9}^{-/-}$ ) did not produce a detectable level of inflammatory cytokines in response to CpG ODN even in the presence of  $\text{IFN}\gamma$ . Further, it was found that the macrophages derived from wild-type mice and TLR9 knockout mice produce almost the same level of  $\text{TNF}\alpha$ , IL-6 and IL-12 in response to LPS or PGN (Fig. 5). Each experimental result shows the average level of  $n=3$ . N.D. in the figures means not detected.

#### 【0054】

Response of spleen cells from wild-type mice (Wild-type) and TLR9 knockout mice ( $\text{TLR9}^{-/-}$ ) against CpG ODN or LPS was also examined. The spleen cells from each mouse ( $1 \times 10^5$ ) were isolated to culture in 96 well plates by CpG DNA or LPS of various concentrations shown in Fig. 6, and the spleen cells were stimulated. 40 hours later from culturing,  $1 \mu\text{Ci}$  of [ $^3\text{H}$ ]-thymidine (Dupont) was added, and then further cultured for 8 hours. The amount of uptaking [ $^3\text{H}$ ]-thymidine was measured by  $\beta$  scintillation counter (Packard) (Fig. 6). The results that

although the spleen cells from wild-type mice promote cell proliferating reactions depending on the amount of administrating CpG ODN or LPS, the spleen cells from TLR9 knockout mice did not show any cell proliferating reaction by CpG ODN even with the stimulus of any concentration of CpG ODN. Further, the amount of expressing Major Histocompatibility Complex (MHC) class II on the surface of B cells derived from wild-type mice in response to CpG ODN was increased. However, such increase of the amount of expressing MHC class II induced by CpG ODN in B cells derived from TLR9 knockout mice was not observed. These facts show that the macrophages or B cells from TLR9 knockout mice specifically lack the response against CpG ODN.

【0055】

Next, it is well known that DNA derived from bacteria comprising CpG ODN potentially stimulates dendritic cells, and supports the development of Th1 cell (EMBO J. 18, 6973-6982, 1999, J. Immunol. 161, 3042-3049, 1998, Proc. Natl. Acad. Sci. USA 96, 9305-9310, 1999). Then, the production of CpG ODN-inducing cytokines and the upregulation of the surface molecule of dendritic cells derived from bone marrow were

examined. The bone marrow cells from wild-type mice (Wild-type) or TLR9 knockout mice (TLR9<sup>-/-</sup>) were cultured with 10ng/ml mouse granulocyte macrophage-colony stimulating factor (Peprotech) in RPMI1640 medium supplemented with 10% fetal bovine serum (J. Exp. Med. 176, 1693-1702, 1992), at day 6 of the culture, immature dendritic cells were harvested and cultured in the presence or absence of 0.1  $\mu$ M CpG ODN or 0.1  $\mu$ g/ml LPS in RPMI1640 medium supplemented with 10% fetal bovine serum for 2 days. After the culture, the concentration of IL-12 p40 in the supernatants was measured by ELISA (Fig. 7). The result shows that the dendritic cells derived from wild-type mice produced IL-12 in response to CpG ODN while the dendritic cells derived from TLR9 knockout mice did not induce the production of IL-12 in response to CpG ODN.

【0056】

After culturing in RPMI supplemented with 10% fetal bovine serum was cultured which contains 10ng/ml mouse granulocyte macrophage-colony stimulating factor (Peprotech), the dendritic cells harvested at day 6 were stained with biotinylated antibodies against CD40, CD80, CD86 or MHC class II, developed

with streptavidine labeled with phycoerythrin (PE; PharMingen). The cells were examined by using a FACSCalibur with CELLQuest software (Becton Dickinson) (Fig. 8). The result shows that stimulation by CpG ODN promotes the expression of CD40, CD80, CD86 and MHC class II on the surface of dendritic cells derived from wild-type mouse while it does not promote the expression of these molecules on the surface of dendritic cells derived from TLR9 knockout mouse by the stimulation of CpG ODN (Fig. 8). The dendritic cells from wild-type mice and from TLR9 knockout mouse show similar responses in response to LPS. This result shows that TLR9 is a receptor essential for cell response to CpG ODN.

#### 【0057】

Example 5 (activation of NF- $\kappa$ B, JNK and IRAK in response to CpG ODN of macrophages derived from TLR9 knockout mice)

It is known that signaling via TLRs activates IRAK, a serine-threonine kinase mediated by MyD88, an adaptor molecule, and subsequently activates MAP kinase and NF- $\kappa$ B (Immunity 11, 115-122, 1999). Whether CpG ODN activates the intracellular signaling or not was examined. The peritoneal macrophages (1

$\times 10^6$  cells) from wild-type and TLR9<sup>-/-</sup> mice in Example 3 were stimulated by 1.0  $\mu$ M of CpG ODN or 1.0  $\mu$ g/ml of LPS from Salmonella minnesota Re-595 for the periods indicated in Fig. 9, nucleoproteins were extracted from the macrophages obtained from each mouse to be incubated together with a specific probe comprising NF- $\kappa$ B DNA-binding sites, electrophoresed, and then visualized by autoradiography (Fig. 9).

【0058】

The result shows that when stimulated by CpG ODN, the macrophages derived from wild-type mice increased NF- $\kappa$ B DNA-binding activity while the macrophages derived from TLR9 knockout mice did not increase NF- $\kappa$ B DNA-binding activity. When stimulated by LPS, the macrophages derived from TLR9 knockout mice and the macrophages derived from the wild-type mice show similar NF- $\kappa$ B activities. The result shows that the macrophages derived from a TLR9 knockout mouse specifically lack NF- $\kappa$ B activity by the induction of CpG ODN. The arrows in the figures indicate the sites of the compounds of NF- $\kappa$ B and specific probes, and the arrowheads indicate the sites of specific probes only.

【0059】

As shown above, the macrophages from wild-type mice and TLR9 knockout mice stimulated by CpG ODN or LPS for the periods indicated in Fig. 10 and Fig. 11 were dissolved into a solvent buffer (a buffer comprising 1.0% Triton X-100, 137mM of NaCl, 20mM of Tris-HCl, 5mM of EDTA, 10% glycerol, 1mM of PMSF, 20  $\mu$ g/ml of aprotinin, 20  $\mu$ g/ml of leupeptin, 1mM of  $\text{Na}_3\text{VO}_4$  and 10mM of  $\beta$ -glycerophosphate at the final concentrations; pH8.0), the cell lysates were immunoprecipitated with anti-JNK antibody (Santa Cruz) or anti-IRAK antibody (Hayashibara Seikagaku Kenkyujo Kabushiki Kaisha). As described in a reference (Immunity 11, 115-122, 1999), the JNK activity and IRAK activity were measured by in vitro kinase assay using GST-c-Jun fusion protein (GST-c-Jun) as a substrate (top figures of Fig 10 and Fig.11; GST-c-Jun, Auto).

【0060】

The cell lysates were separated by SDS-polyacrylamide gel electrophoresis to transfer them onto a nitrocellulose membrane and blotted the membrane with anti-JNK antibody (Santa Cruz) or anti-IRAK antibody (Transduction Laboratories) to visualize



using an enhanced chemiluminescent system (Dupont) (bottom figures of Fig. 10 and Fig. 11; WB). The result shows that CpG ODN activates JUN and IRAK of the macrophages derived from wild-type mice while it does not activate JUN and IRAK of the macrophages derived from TLR9 knockout mice (Fig. 10 and Fig. 11). It is therefore found that the signaling transduction mediated by CpG ODN depends on TLR9.

【0061】

#### 【Effects of the Invention】

Bacteria-derived DNA comprising an unmethylated CpG motif significantly activates immune cells and induce Th1 response, while a receptor recognizing such bacterial DNA remained unknown. The present invention has revealed a receptor of oligonucleotides comprising an unmethylated CpG sequence of bacterial DNA and will enable us to elucidate a receptor protein TLR9, a member of TLR family, specifically recognizing bacterial DNA having an unmethylated CpG sequence, the genetic DNA encoding it or others, which will be useful to diagnose and treat bacterial diseases and others. The use of the TLR9 knockout animals will also enable us to elucidate functional mechanisms of DNA derived

from bacteria at the molecular level.

【0062】

【Sequence Listing】

SEQUENCE LISTING

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Met Gly Phe	
1	
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Cys Arg Ser Ala Leu His Pro Leu Ser Leu Leu Val Gln Ala Ile Met	
5 10 15	
ctg gcc atg acc ctg gcc ctg ggt acc ttg cct gcc ttc cta ccc tgt	211
Leu Ala Met Thr Leu Ala Leu Gly Thr Leu Pro Ala Phe Leu Pro Cys	
20 25 30 35	
gag ctc cag ccc cac ggc ctg gtg aac tgc aac tgg ctg ttc ctg aag	259
Glu Leu Gln Pro His Gly Leu Val Asn Cys Asn Trp Leu Phe Leu Lys	
40 45 50	
tct gtg ccc cac ttc tcc atg gca gca ccc cgt ggc aat gtc acc agc	307
Ser Val Pro His Phe Ser Met Ala Ala Pro Arg Gly Asn Val Thr Ser	
55 60 65	
ctt tcc ttg tcc tcc aac cgc atc cac cac ctc cat gat tct gac ttt	355
Leu Ser Leu Ser Ser Asn Arg Ile His His Leu His Asp Ser Asp Phe	
70 75 80	
gcc cac ctg ccc agc ctg cgg cat ctc aac ctc aag tgg aac tgc ccg	403
Ala His Leu Pro Ser Leu Arg His Leu Asn Leu Lys Trp Asn Cys Pro	
85 90 95	
ccg gtt ggc ctc agc ccc atg cac ttc ccc tgc cac atg acc atc gag	451
Pro Val Gly Leu Ser Pro Met His Phe Pro Cys His Met Thr Ile Glu	

100	105	110	115	
ccc agc acc ttc ttg gct gtg ccc acc ctg gaa gag cta aac ctg agc				499
Pro Ser Thr Phe Leu Ala Val Pro Thr Leu Glu Glu Leu Asn Leu Ser				
	120	125	130	
tac aac aac atc atg act gtg cct gcg ctg ccc aaa tcc ctc ata tcc				547
Tyr Asn Asn Ile Met Thr Val Pro Ala Leu Pro Lys Ser Leu Ile Ser				
	135	140	145	
ctg tcc ctc agc cat acc aac atc ctg atg cta gac tct gcc agc ctc				595
Leu Ser Leu Ser His Thr Asn Ile Leu Met Leu Asp Ser Ala Ser Leu				
	150	155	160	
gcc ggc ctg cat gcc ctg cgc ttc cta ttc atg gac ggc aac tgt tat				643
Ala Gly Leu His Ala Leu Arg Phe Leu Phe Met Asp Gly Asn Cys Tyr				
	165	170	175	
tac aag aac ccc tgc agg cag gca ctg gag gtg gcc ccg ggt gcc ctc				691
Tyr Lys Asn Pro Cys Arg Gln Ala Leu Glu Val Ala Pro Gly Ala Leu				
180	185	190	195	
ctt ggc ctg ggc aac ctc acc cac ctg tca ctc aag tac aac aac ctc				739
Leu Gly Leu Gly Asn Leu Thr His Leu Ser Leu Lys Tyr Asn Asn Leu				
	200	205	210	
act gtg gtg ccc cgc aac ctg cct tcc agc ctg gag tat ctg ctg ttg				787
Thr Val Val Pro Arg Asn Leu Pro Ser Ser Leu Glu Tyr Leu Leu Leu				
	215	220	225	

tcc tac aac cgc atc gtc aaa ctg gcg cct gag gac ctg gcc aat ctg	835
Ser Tyr Asn Arg Ile Val Lys Leu Ala Pro Glu Asp Leu Ala Asn Leu	
230 235 240	
acc gcc ctg cgt gtg ctc gat gtg ggc gga aat tgc cgc cgc tgc gac	883
Thr Ala Leu Arg Val Leu Asp Val Gly Gly Asn Cys Arg Arg Cys Asp	
245 250 255	
cac gct ccc aac ccc tgc atg gag tgc cct cgt cac ttc ccc cag cta	931
His Ala Pro Asn Pro Cys Met Glu Cys Pro Arg His Phe Pro Gln Leu	
260 265 270 275	
cat ccc gat acc ttc agc cac ctg agc cgt ctt gaa ggc ctg gtg ttg	979
His Pro Asp Thr Phe Ser His Leu Ser Arg Leu Glu Gly Leu Val Leu	
280 285 290	
aag gac agt tct ctc tcc tgg ctg aat gcc agt tgg ttc cgt ggg ctg	1027
Lys Asp Ser Ser Leu Ser Trp Leu Asn Ala Ser Trp Phe Arg Gly Leu	
295 300 305	
gga aac ctc cga gtg ctg gac ctg agt gag aac ttc ctc tac aaa tgc	1075
Gly Asn Leu Arg Val Leu Asp Leu Ser Glu Asn Phe Leu Tyr Lys Cys	
310 315 320	
atc act aaa acc aag gcc ttc cag ggc cta aca cag ctg cgc aag ctt	1123
Ile Thr Lys Thr Lys Ala Phe Gln Gly Leu Thr Gln Leu Arg Lys Leu	
325 330 335	
aac ctg tcc ttc aat tac caa aag agg gtg tcc ttt gcc cac ctg tct	1171
Asn Leu Ser Phe Asn Tyr Gln Lys Arg Val Ser Phe Ala His Leu Ser	

340	345	350	355	
ctg gcc cct tcc ttc ggg agc ctg gtc gcc ctg aag gag ctg gac atg				1219
Leu Ala Pro Ser Phe Gly Ser Leu Val Ala Leu Lys Glu Leu Asp Met				
	360	365	370	
cac ggc atc ttc ttc cgc tca ctc gat gag acc acg ctc cgg cca ctg				1267
His Gly Ile Phe Phe Arg Ser Leu Asp Glu Thr Thr Leu Arg Pro Leu				
	375	380	385	
gcc cgc ctg ccc atg ctc cag act ctg cgt ctg cag atg aac ttc atc				1315
Ala Arg Leu Pro Met Leu Gln Thr Leu Arg Leu Gln Met Asn Phe Ile				
	390	395	400	
aac cag gcc cag ctc ggc atc ttc agg gcc ttc cct ggc ctg cgc tac				1363
Asn Gln Ala Gln Leu Gly Ile Phe Arg Ala Phe Pro Gly Leu Arg Tyr				
	405	410	415	
gtg gac ctg tcg gac aac cgc atc agc gga gct tcg gag ctg aca gcc				1411
Val Asp Leu Ser Asp Asn Arg Ile Ser Gly Ala Ser Glu Leu Thr Ala				
	420	425	430	435
acc atg ggg gag gca gat gga ggg gag aag gtc tgg ctg cag cct ggg				1459
Thr Met Gly Glu Ala Asp Gly Gly Glu Lys Val Trp Leu Gln Pro Gly				
	440	445	450	
gac ctt gct ccg gcc cca gtg gac act ccc agc tct gaa gac ttc agg				1507
Asp Leu Ala Pro Ala Pro Val Asp Thr Pro Ser Ser Glu Asp Phe Arg				
	455	460	465	

ccc aac tgc agc acc ctc aac ttc acc ttg gat ctg tca cgg aac aac 1555  
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Leu Val Thr Val Gln Pro Glu Met Phe Ala Gln Leu Ser His Leu Gln  
485 490 495

tgc ctg cgc ctg agc cac aac tgc atc tcg cag gca gtc aat ggc tcc 1651  
Cys Leu Arg Leu Ser His Asn Cys Ile Ser Gln Ala Val Asn Gly Ser  
500 505 510 515

cag ttc ctg ccg ctg acc ggt ctg cag gtg cta gac ctg tcc cac aat 1699  
Gln Phe Leu Pro Leu Thr Gly Leu Gln Val Leu Asp Leu Ser His Asn  
520 525 530

aag ctg gac ctc tac cac gag cac tca ttc acg gag cta cca cga ctg 1747  
Lys Leu Asp Leu Tyr His Glu His Ser Phe Thr Glu Leu Pro Arg Leu  
535 540 545

gag gcc ctg gac ctc agc tac aac agc cag ccc ttt ggc atg cag ggc 1795  
Glu Ala Leu Asp Leu Ser Tyr Asn Ser Gln Pro Phe Gly Met Gln Gly  
550 555 560

gtg ggc cac aac ttc agc ttc gtg gct cac ctg cgc acc ctg cgc cac 1843  
Val Gly His Asn Phe Ser Phe Val Ala His Leu Arg Thr Leu Arg His  
565 570 575

ctc agc ctg gcc cac aac aac atc cac agc caa gtg tcc cag cag ctc 1891  
Leu Ser Leu Ala His Asn Asn Ile His Ser Gln Val Ser Gln Gln Leu



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tgc agt acg tcg ctg cgg gcc ctg gac ttc agc ggc aat gca ctg ggc	1939			
Cys Ser Thr Ser Leu Arg Ala Leu Asp Phe Ser Gly Asn Ala Leu Gly				
600	605	610		
cat atg tgg gcc gag gga gac ctc tat ctg cac ttc ttc caa ggc ctg	1987			
His Met Trp Ala Glu Gly Asp Leu Tyr Leu His Phe Phe Gln Gly Leu				
615	620	625		
agc ggt ttg atc tgg ctg gac ttg tcc cag aac cgc ctg cac acc ctc	2035			
Ser Gly Leu Ile Trp Leu Asp Leu Ser Gln Asn Arg Leu His Thr Leu				
630	635	640		
ctg ccc caa acc ctg cgc aac ctc ccc aag agc cta cag gtg ctg cgt	2083			
Leu Pro Gln Thr Leu Arg Asn Leu Pro Lys Ser Leu Gln Val Leu Arg				
645	650	655		
ctc cgt gac aat tac ctg gcc ttc ttt aag tgg tgg agc ctc cac ttc	2131			
Leu Arg Asp Asn Tyr Leu Ala Phe Phe Lys Trp Trp Ser Leu His Phe				
660	665	670	675	
ctg ccc aaa ctg gaa gtc ctc gac ctg gca gga aac cag ctg aag gcc	2179			
Leu Pro Lys Leu Glu Val Leu Asp Leu Ala Gly Asn Gln Leu Lys Ala				
680	685	690		
ctg acc aat ggc agc ctg cct gct ggc acc cgg ctc cgg agg ctg gat	2227			
Leu Thr Asn Gly Ser Leu Pro Ala Gly Thr Arg Leu Arg Arg Leu Asp				
695	700	705		

gtc agc tgc aac agc atc agc ttc gtg gcc ccc ggc ttc ttt tcc aag	2275
Val Ser Cys Asn Ser Ile Ser Phe Val Ala Pro Gly Phe Phe Ser Lys	
710 715 720	
gcc aag gag ctg cga gag ctc aac ctt agc gcc aac gcc ctc aag aca	2323
Ala Lys Glu Leu Arg Glu Leu Asn Leu Ser Ala Asn Ala Leu Lys Thr	
725 730 735	
gtg gac cac tcc tgg ttt ggg ccc ctg gcg agt gcc ctg caa ata cta	2371
Val Asp His Ser Trp Phe Gly Pro Leu Ala Ser Ala Leu Gln Ile Leu	
740 745 750 755	
gat gta agc gcc aac cct ctg cac tgc gcc tgt ggg gcg gcc ttt atg	2419
Asp Val Ser Ala Asn Pro Leu His Cys Ala Cys Gly Ala Ala Phe Met	
760 765 770	
gac ttc ctg ctg gag gtg cag gct gcc gtg ccc ggt ctg ccc agc cgg	2467
Asp Phe Leu Leu Glu Val Gln Ala Ala Val Pro Gly Leu Pro Ser Arg	
775 780 785	
gtg aag tgt ggc agt ccg ggc cag ctc cag ggc ctc agc atc ttt gca	2515
Val Lys Cys Gly Ser Pro Gly Gln Leu Gln Gly Leu Ser Ile Phe Ala	
790 795 800	
cag gac ctg cgc ctc tgc ctg gat gag gcc ctc tcc tgg gac tgt ttc	2563
Gln Asp Leu Arg Leu Cys Leu Asp Glu Ala Leu Ser Trp Asp Cys Phe	
805 810 815	
gcc ctc tcg ctg ctg gct gtg gct ctg ggc ctg ggt gtg ccc atg ctg	2611
Ala Leu Ser Leu Leu Ala Val Ala Leu Gly Leu Gly Val Pro Met Leu	

820	825	830	835	
cat cac ctc tgt ggc tgg gac ctc tgg tac tgc ttc cac ctg tgc ctg				2659
His His Leu Cys Gly Trp Asp Leu Trp Tyr Cys Phe His Leu Cys Leu				
	840	845	850	
gcc tgg ctt ccc tgg cgg ggg cgg caa agt ggg cga gat gag gat gcc				2707
Ala Trp Leu Pro Trp Arg Gly Arg Gln Ser Gly Arg Asp Glu Asp Ala				
	855	860	865	
ctg ccc tac gat gcc ttc gtg gtc ttc gac aaa acg cag agc gca gtg				2755
Leu Pro Tyr Asp Ala Phe Val Val Phe Asp Lys Thr Gln Ser Ala Val				
	870	875	880	
gca gac tgg gtg tac aac gag ctt cgg ggg cag ctg gag gag tgc cgt				2803
Ala Asp Trp Val Tyr Asn Glu Leu Arg Gly Gln Leu Glu Glu Cys Arg				
	885	890	895	
ggg cgc tgg gca ctc cgc ctg tgc ctg gag gaa cgc gac tgg ctg cct				2851
Gly Arg Trp Ala Leu Arg Leu Cys Leu Glu Glu Arg Asp Trp Leu Pro				
900	905	910	915	
ggc aaa acc ctc ttt gag aac ctg tgg gcc tcg gtc tat ggc agc cgc				2899
Gly Lys Thr Leu Phe Glu Asn Leu Trp Ala Ser Val Tyr Gly Ser Arg				
	920	925	930	
aag acg ctg ttt gtg ctg gcc cac acg gac cgg gtc agt ggt ctc ttg				2947
Lys Thr Leu Phe Val Leu Ala His Thr Asp Arg Val Ser Gly Leu Leu				
	935	940	945	

cgc gcc agc ttc ctg ctg gcc cag cag cgc ctg ctg gag gac cgc aag 2995  
 Arg Ala Ser Phe Leu Leu Ala Gln Gln Arg Leu Leu Glu Asp Arg Lys

950 955 960

gac gtc gtg gtg ctg gtg atc ctg agc cct gac ggc cgc cgc tcc cgc 3043  
 Asp Val Val Val Leu Val Ile Leu Ser Pro Asp Gly Arg Arg Ser Arg

965 970 975

tac gtg cgg ctg cgc cag cgc ctc tgc cgc cag agt gtc ctc ctc tgg 3091  
 Tyr Val Arg Leu Arg Gln Arg Leu Cys Arg Gln Ser Val Leu Leu Trp

980 985 990 995

ccc cac cag ccc agt ggt cag cgc agc ttc tgg gcc cag ctg ggc atg 3139  
 Pro His Gln Pro Ser Gly Gln Arg Ser Phe Trp Ala Gln Leu Gly Met

1000 1005 1010

gcc ctg acc agg gac aac cac cac ttc tat aac cgg aac ttc tgc cag 3187  
 Ala Leu Thr Arg Asp Asn His His Phe Tyr Asn Arg Asn Phe Cys Gln

1015 1020 1025

gga ccc acg gcc gaa tag ccgtgagccg gaatcctgca cgggtgccacc 3235  
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1030

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Ala	Ile	Met	Leu	Ala	Met	Thr	Leu	Ala	Leu	Gly	Thr	Leu	Pro	Ala	Phe
			20					25					30		
Leu	Pro	Cys	Glu	Leu	Gln	Pro	His	Gly	Leu	Val	Asn	Cys	Asn	Trp	Leu
		35					40					45			
Phe	Leu	Lys	Ser	Val	Pro	His	Phe	Ser	Met	Ala	Ala	Pro	Arg	Gly	Asn
	50					55					60				
Val	Thr	Ser	Leu	Ser	Leu	Ser	Ser	Asn	Arg	Ile	His	His	Leu	His	Asp
65				70					75					80	
Ser	Asp	Phe	Ala	His	Leu	Pro	Ser	Leu	Arg	His	Leu	Asn	Leu	Lys	Trp
				85					90					95	
Asn	Cys	Pro	Pro	Val	Gly	Leu	Ser	Pro	Met	His	Phe	Pro	Cys	His	Met
		100						105					110		
Thr	Ile	Glu	Pro	Ser	Thr	Phe	Leu	Ala	Val	Pro	Thr	Leu	Glu	Glu	Leu
	115						120					125			
Asn	Leu	Ser	Tyr	Asn	Asn	Ile	Met	Thr	Val	Pro	Ala	Leu	Pro	Lys	Ser
	130					135					140				
Leu	Ile	Ser	Leu	Ser	Leu	Ser	His	Thr	Asn	Ile	Leu	Met	Leu	Asp	Ser
145				150					155					160	
Ala	Ser	Leu	Ala	Gly	Leu	His	Ala	Leu	Arg	Phe	Leu	Phe	Met	Asp	Gly
			165					170					175		
Asn	Cys	Tyr	Tyr	Lys	Asn	Pro	Cys	Arg	Gln	Ala	Leu	Glu	Val	Ala	Pro
		180						185					190		
Gly	Ala	Leu	Leu	Gly	Leu	Gly	Asn	Leu	Thr	His	Leu	Ser	Leu	Lys	Tyr
	195						200					205			
Asn	Asn	Leu	Thr	Val	Val	Pro	Arg	Asn	Leu	Pro	Ser	Ser	Leu	Glu	Tyr

210	215	220
Leu Leu Leu Ser Tyr Asn Arg Ile Val Lys Leu Ala Pro Glu Asp Leu		
225	230	235
Ala Asn Leu Thr Ala Leu Arg Val Leu Asp Val Gly Gly Asn Cys Arg		
245	250	255
Arg Cys Asp His Ala Pro Asn Pro Cys Met Glu Cys Pro Arg His Phe		
260	265	270
Pro Gln Leu His Pro Asp Thr Phe Ser His Leu Ser Arg Leu Glu Gly		
275	280	285
Leu Val Leu Lys Asp Ser Ser Leu Ser Trp Leu Asn Ala Ser Trp Phe		
290	295	300
Arg Gly Leu Gly Asn Leu Arg Val Leu Asp Leu Ser Glu Asn Phe Leu		
305	310	315
Tyr Lys Cys Ile Thr Lys Thr Lys Ala Phe Gln Gly Leu Thr Gln Leu		
325	330	335
Arg Lys Leu Asn Leu Ser Phe Asn Tyr Gln Lys Arg Val Ser Phe Ala		
340	345	350
His Leu Ser Leu Ala Pro Ser Phe Gly Ser Leu Val Ala Leu Lys Glu		
355	360	365
Leu Asp Met His Gly Ile Phe Phe Arg Ser Leu Asp Glu Thr Thr Leu		
370	375	380
Arg Pro Leu Ala Arg Leu Pro Met Leu Gln Thr Leu Arg Leu Gln Met		
385	390	395
Asn Phe Ile Asn Gln Ala Gln Leu Gly Ile Phe Arg Ala Phe Pro Gly		
405	410	415
Leu Arg Tyr Val Asp Leu Ser Asp Asn Arg Ile Ser Gly Ala Ser Glu		
420	425	430
Leu Thr Ala Thr Met Gly Glu Ala Asp Gly Gly Glu Lys Val Trp Leu		
435	440	445
Gln Pro Gly Asp Leu Ala Pro Ala Pro Val Asp Thr Pro Ser Ser Glu		

450	455	460
Asp Phe Arg Pro Asn Cys Ser Thr Leu Asn Phe Thr Leu Asp Leu Ser		
465	470	475
Arg Asn Asn Leu Val Thr Val Gln Pro Glu Met Phe Ala Gln Leu Ser		
485	490	495
His Leu Gln Cys Leu Arg Leu Ser His Asn Cys Ile Ser Gln Ala Val		
500	505	510
Asn Gly Ser Gln Phe Leu Pro Leu Thr Gly Leu Gln Val Leu Asp Leu		
515	520	525
Ser His Asn Lys Leu Asp Leu Tyr His Glu His Ser Phe Thr Glu Leu		
530	535	540
Pro Arg Leu Glu Ala Leu Asp Leu Ser Tyr Asn Ser Gln Pro Phe Gly		
545	550	555
Met Gln Gly Val Gly His Asn Phe Ser Phe Val Ala His Leu Arg Thr		
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Leu Arg His Leu Ser Leu Ala His Asn Asn Ile His Ser Gln Val Ser		
580	585	590
Gln Gln Leu Cys Ser Thr Ser Leu Arg Ala Leu Asp Phe Ser Gly Asn		
595	600	605
Ala Leu Gly His Met Trp Ala Glu Gly Asp Leu Tyr Leu His Phe Phe		
610	615	620
Gln Gly Leu Ser Gly Leu Ile Trp Leu Asp Leu Ser Gln Asn Arg Leu		
625	630	635
His Thr Leu Leu Pro Gln Thr Leu Arg Asn Leu Pro Lys Ser Leu Gln		
645	650	655
Val Leu Arg Leu Arg Asp Asn Tyr Leu Ala Phe Phe Lys Trp Trp Ser		
660	665	670
Leu His Phe Leu Pro Lys Leu Glu Val Leu Asp Leu Ala Gly Asn Gln		
675	680	685
Leu Lys Ala Leu Thr Asn Gly Ser Leu Pro Ala Gly Thr Arg Leu Arg		

690	695	700
Arg Leu Asp Val Ser Cys Asn Ser Ile Ser Phe Val Ala Pro Gly Phe		
705	710	715
Phe Ser Lys Ala Lys Glu Leu Arg Glu Leu Asn Leu Ser Ala Asn Ala		720
	725	730
Leu Lys Thr Val Asp His Ser Trp Phe Gly Pro Leu Ala Ser Ala Leu		735
	740	745
Gln Ile Leu Asp Val Ser Ala Asn Pro Leu His Cys Ala Cys Gly Ala		750
	755	760
Ala Phe Met Asp Phe Leu Leu Glu Val Gln Ala Ala Val Pro Gly Leu		765
	770	775
Pro Ser Arg Val Lys Cys Gly Ser Pro Gly Gln Leu Gln Gly Leu Ser		780
785	790	795
Ile Phe Ala Gln Asp Leu Arg Leu Cys Leu Asp Glu Ala Leu Ser Trp		800
	805	810
Asp Cys Phe Ala Leu Ser Leu Leu Ala Val Ala Leu Gly Leu Gly Val		815
	820	825
Pro Met Leu His His Leu Cys Gly Trp Asp Leu Trp Tyr Cys Phe His		830
	835	840
Leu Cys Leu Ala Trp Leu Pro Trp Arg Gly Arg Gln Ser Gly Arg Asp		845
	850	855
Glu Asp Ala Leu Pro Tyr Asp Ala Phe Val Val Phe Asp Lys Thr Gln		860
865	870	875
Ser Ala Val Ala Asp Trp Val Tyr Asn Glu Leu Arg Gly Gln Leu Glu		880
	885	890
Glu Cys Arg Gly Arg Trp Ala Leu Arg Leu Cys Leu Glu Glu Arg Asp		895
	900	905
Trp Leu Pro Gly Lys Thr Leu Phe Glu Asn Leu Trp Ala Ser Val Tyr		910
	915	920
Gly Ser Arg Lys Thr Leu Phe Val Leu Ala His Thr Asp Arg Val Ser		925



930	935	940	
Gly Leu Leu Arg Ala Ser Phe Leu Leu Ala Gln Gln Arg Leu Leu Glu			
945	950	955	960
Asp Arg Lys Asp Val Val Val Leu Val Ile Leu Ser Pro Asp Gly Arg			
	965	970	975
Arg Ser Arg Tyr Val Arg Leu Arg Gln Arg Leu Cys Arg Gln Ser Val			
	980	985	990
Leu Leu Trp Pro His Gln Pro Ser Gly Gln Arg Ser Phe Trp Ala Gln			
	995	1000	1005
Leu Gly Met Ala Leu Thr Arg Asp Asn His His Phe Tyr Asn Arg Asn			
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Met Val Leu

cgt cga agg act ctg cac ccc ttg tcc ctc ctg gta cag gct gca gtg	163
Arg Arg Arg Thr Leu His Pro Leu Ser Leu Leu Val Gln Ala Ala Val	
5 10 15	
ctg gct gag act ctg gcc ctg ggt acc ctg cct gcc ttc cta ccc tgt	211
Leu Ala Glu Thr Leu Ala Leu Gly Thr Leu Pro Ala Phe Leu Pro Cys	
20 25 30 35	
gag ctg aag cct cat ggc ctg gtg gac tgc aat tgg ctg ttc ctg aag	259
Glu Leu Lys Pro His Gly Leu Val Asp Cys Asn Trp Leu Phe Leu Lys	
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tct gta ccc cgt ttc tct gcg gca gca tcc tgc tcc aac atc acc cgc	307
Ser Val Pro Arg Phe Ser Ala Ala Ala Ser Cys Ser Asn Ile Thr Arg	
55 60 65	
ctc tcc ttg atc tcc aac cgt atc cac cac ctg cac aac tcc gac ttc	355
Leu Ser Leu Ile Ser Asn Arg Ile His His Leu His Asn Ser Asp Phe	
70 75 80	
gtc cac ctg tcc aac ctg cgg cag ctg aac ctc aag tgg aac tgt cca	403
Val His Leu Ser Asn Leu Arg Gln Leu Asn Leu Lys Trp Asn Cys Pro	
85 90 95	
ccc act ggc ctt agc ccc ttg cac ttc tct tgc cac atg acc att gag	451
Pro Thr Gly Leu Ser Pro Leu His Phe Ser Cys His Met Thr Ile Glu	
100 105 110 115	

ccc aga acc ttc ctg gct atg cgt aca ctg gag gag ctg aac ctg agc	499
Pro Arg Thr Phe Leu Ala Met Arg Thr Leu Glu Glu Leu Asn Leu Ser	
120 125 130	
tat aat ggt atc acc act gtg ccc cga ctg ccc agc tcc ctg gtg aat	547
Tyr Asn Gly Ile Thr Thr Val Pro Arg Leu Pro Ser Ser Leu Val Asn	
135 140 145	
ctg agc ctg agc cac acc aac atc ctg gtt cta gat gct aac agc ctc	595
Leu Ser Leu Ser His Thr Asn Ile Leu Val Leu Asp Ala Asn Ser Leu	
150 155 160	
gcc ggc cta tac agc ctg cgc gtt ctc ttc atg gac ggg aac tgc tac	643
Ala Gly Leu Tyr Ser Leu Arg Val Leu Phe Met Asp Gly Asn Cys Tyr	
165 170 175	
tac aag aac ccc tgc aca gga gcg gtg aag gtg acc cca ggc gcc ctc	691
Tyr Lys Asn Pro Cys Thr Gly Ala Val Lys Val Thr Pro Gly Ala Leu	
180 185 190 195	
ctg ggc ctg agc aat ctc acc cat ctg tct gtg aag tat aac aac ctc	739
Leu Gly Leu Ser Asn Leu Thr His Leu Ser Val Lys Tyr Asn Asn Leu	
200 205 210	
aca aag gtg ccc cgc caa ctg ccc ccc agc ctg gag tac ctc ctg gtg	787
Thr Lys Val Pro Arg Gln Leu Pro Pro Ser Leu Glu Tyr Leu Leu Val	
215 220 225	
tcc tat aac ctc att gtc aag ctg ggg cct gaa gac ctg gcc aat ctg	835
Ser Tyr Asn Leu Ile Val Lys Leu Gly Pro Glu Asp Leu Ala Asn Leu	

230	235	240	
acc tcc ctt cga gta ctt gat gtg ggt ggg aat tgc cgt cgc tgc gac			883
Thr Ser Leu Arg Val Leu Asp Val Gly Gly Asn Cys Arg Arg Cys Asp			
245	250	255	
cat gcc ccc aat ccc tgt ata gaa tgt ggc caa aag tcc ctc cac ctg			931
His Ala Pro Asn Pro Cys Ile Glu Cys Gly Gln Lys Ser Leu His Leu			
260	265	270	275
cac cct gag acc ttc cat cac ctg agc cat ctg gaa ggc ctg gtg ctg			979
His Pro Glu Thr Phe His His Leu Ser His Leu Glu Gly Leu Val Leu			
280	285	290	
aag gac agc tct ctc cat aca ctg aac tct tcc tgg ttc caa ggt ctg			1027
Lys Asp Ser Ser Leu His Thr Leu Asn Ser Ser Trp Phe Gln Gly Leu			
295	300	305	
gtc aac ctc tcg gtg ctg gac cta agc gag aac ttt ctc tat gaa agc			1075
Val Asn Leu Ser Val Leu Asp Leu Ser Glu Asn Phe Leu Tyr Glu Ser			
310	315	320	
atc aac cac acc aat gcc ttt cag aac cta acc cgc ctg cgc aag ctc			1123
Ile Asn His Thr Asn Ala Phe Gln Asn Leu Thr Arg Leu Arg Lys Leu			
325	330	335	
aac ctg tcc ttc aat tac cgc aag aag gta tcc ttt gcc cgc ctc cac			1171
Asn Leu Ser Phe Asn Tyr Arg Lys Lys Val Ser Phe Ala Arg Leu His			
340	345	350	355

ctg gca agt tcc ttc aag aac ctg gtg tca ctg cag gag ctg aac atg	1219
Leu Ala Ser Ser Phe Lys Asn Leu Val Ser Leu Gln Glu Leu Asn Met	
360 365 370	
aac ggc atc ttc ttc cgc tgc ctc aac aag tac acg ctc aga tgg ctg	1267
Asn Gly Ile Phe Phe Arg Ser Leu Asn Lys Tyr Thr Leu Arg Trp Leu	
375 380 385	
gcc gat ctg ccc aaa ctc cac act ctg cat ctt caa atg aac ttc atc	1315
Ala Asp Leu Pro Lys Leu His Thr Leu His Leu Gln Met Asn Phe Ile	
390 395 400	
aac cag gca cag ctc agc atc ttt ggt acc ttc cga gcc ctt cgc ttt	1363
Asn Gln Ala Gln Leu Ser Ile Phe Gly Thr Phe Arg Ala Leu Arg Phe	
405 410 415	
gtg gac ttg tca gac aat cgc atc agt ggg cct tca acg ctg tca gaa	1411
Val Asp Leu Ser Asp Asn Arg Ile Ser Gly Pro Ser Thr Leu Ser Glu	
420 425 430 435	
gcc acc cct gaa gag gca gat gat gca gag cag gag gag ctg ttg tct	1459
Ala Thr Pro Glu Glu Ala Asp Asp Ala Glu Gln Glu Glu Leu Leu Ser	
440 445 450	
gcg gat cct cac cca gct cca ctg agc acc cct gct tct aag aac ttc	1507
Ala Asp Pro His Pro Ala Pro Leu Ser Thr Pro Ala Ser Lys Asn Phe	
455 460 465	
atg gac agg tgt aag aac ttc aag ttc acc atg gac ctg tct cgg aac	1555
Met Asp Arg Cys Lys Asn Phe Lys Phe Thr Met Asp Leu Ser Arg Asn	

470	475	480	
aac ctg gtg act atc aag cca gag atg ttt gtc aat ctc tca cgc ctc			1603
Asn Leu Val Thr Ile Lys Pro Glu Met Phe Val Asn Leu Ser Arg Leu			
485	490	495	
cag tgt ctt agc ctg agc cac aac tcc att gca cag gct gtc aat ggc			1651
Gln Cys Leu Ser Leu Ser His Asn Ser Ile Ala Gln Ala Val Asn Gly			
500	505	510	515
tct cag ttc ctg ccg ctg act aat ctg cag gtg ctg gac ctg tcc cat			1699
Ser Gln Phe Leu Pro Leu Thr Asn Leu Gln Val Leu Asp Leu Ser His			
520	525	530	
aac aaa ctg gac ttg tac cac tgg aaa tcg ttc agt gag cta cca cag			1747
Asn Lys Leu Asp Leu Tyr His Trp Lys Ser Phe Ser Glu Leu Pro Gln			
535	540	545	
ttg cag gcc ctg gac ctg agc tac aac agc cag ccc ttt agc atg aag			1795
Leu Gln Ala Leu Asp Leu Ser Tyr Asn Ser Gln Pro Phe Ser Met Lys			
550	555	560	
ggt ata ggc cac aat ttc agt ttt gtg gcc cat ctg tcc atg cta cac			1843
Gly Ile Gly His Asn Phe Ser Phe Val Ala His Leu Ser Met Leu His			
565	570	575	
agc ctt agc ctg gca cac aat gac att cat acc cgt gtg tcc tca cat			1891
Ser Leu Ser Leu Ala His Asn Asp Ile His Thr Arg Val Ser Ser His			
580	585	590	595

ctc aac agc aac tca gtg agg ttt ctt gac ttc agc ggc aac ggt atg	1939
Leu Asn Ser Asn Ser Val Arg Phe Leu Asp Phe Ser Gly Asn Gly Met	
600 605 610	
ggc cgc atg tgg gat gag ggg ggc ctt tat ctc cat ttc ttc caa ggc	1987
Gly Arg Met Trp Asp Glu Gly Gly Leu Tyr Leu His Phe Phe Gln Gly	
615 620 625	
ctg agt ggc ctg ctg aag ctg gac ctg tct caa aat aac ctg cat atc	2035
Leu Ser Gly Leu Leu Lys Leu Asp Leu Ser Gln Asn Asn Leu His Ile	
630 635 640	
ctc cgg ccc cag aac ctt gac aac ctc ccc aag agc ctg aag ctg ctg	2083
Leu Arg Pro Gln Asn Leu Asp Asn Leu Pro Lys Ser Leu Lys Leu Leu	
645 650 655	
agc ctc cga gac aac tac cta tct ttc ttt aac tgg acc agt ctg tcc	2131
Ser Leu Arg Asp Asn Tyr Leu Ser Phe Phe Asn Trp Thr Ser Leu Ser	
660 665 670 675	
ttc ctg ccc aac ctg gaa gtc cta gac ctg gca ggc aac cag cta aag	2179
Phe Leu Pro Asn Leu Glu Val Leu Asp Leu Ala Gly Asn Gln Leu Lys	
680 685 690	
gcc ctg acc aat ggc acc ctg cct aat ggc acc ctc ctc cag aaa ctg	2227
Ala Leu Thr Asn Gly Thr Leu Pro Asn Gly Thr Leu Leu Gln Lys Leu	
695 700 705	
gat gtc agc agc aac agt atc gtc tct gtg gtc cca gcc ttc ttc gct	2275
Asp Val Ser Ser Asn Ser Ile Val Ser Val Val Pro Ala Phe Phe Ala	

710	715	720	
ctg gcg gtc gag ctg aaa gag gtc aac ctc agc cac aac att ctc aag	2323		
Leu Ala Val Glu Leu Lys Glu Val Asn Leu Ser His Asn Ile Leu Lys			
725	730	735	
acg gtg gat cgc tcc tgg ttt ggg ccc att gtg atg aac ctg aca gtt	2371		
Thr Val Asp Arg Ser Trp Phe Gly Pro Ile Val Met Asn Leu Thr Val			
740	745	750	755
cta gac gtg aga agc aac cct ctg cac tgt gcc tgt ggg gca gcc ttc	2419		
Leu Asp Val Arg Ser Asn Pro Leu His Cys Ala Cys Gly Ala Ala Phe			
760	765	770	
gta gac tta ctg ttg gag gtg cag acc aag gtg cct ggc ctg gct aat	2467		
Val Asp Leu Leu Leu Glu Val Gln Thr Lys Val Pro Gly Leu Ala Asn			
775	780	785	
ggt gtg aag tgt ggc agc ccc ggc cag ctg cag ggc cgt agc atc ttc	2515		
Gly Val Lys Cys Gly Ser Pro Gly Gln Leu Gln Gly Arg Ser Ile Phe			
790	795	800	
gca cag gac ctg cgg ctg tgc ctg gat gag gtc ctc tct tgg gac tgc	2563		
Ala Gln Asp Leu Arg Leu Cys Leu Asp Glu Val Leu Ser Trp Asp Cys			
805	810	815	
ttt ggc ctt tca ctc ttg gct gtg gcc gtg ggc atg gtg gtg cct ata	2611		
Phe Gly Leu Ser Leu Leu Ala Val Ala Val Gly Met Val Val Pro Ile			
820	825	830	835



ctg cac cat ctc tgc ggc tgg gac gtc tgg tac tgt ttt cat ctg tgc	2659
Leu His His Leu Cys Gly Trp Asp Val Trp Tyr Cys Phe His Leu Cys	
840 845 850	
ctg gca tgg cta cct ttg ctg gcc cgc agc cga cgc agc gcc caa gct	2707
Leu Ala Trp Leu Pro Leu Leu Ala Arg Ser Arg Arg Ser Ala Gln Ala	
855 860 865	
ctc ccc tat gat gcc ttc gtg gtg ttc gat aag gca cag agc gca gtt	2755
Leu Pro Tyr Asp Ala Phe Val Val Phe Asp Lys Ala Gln Ser Ala Val	
870 875 880	
gcg gac tgg gtg tat aac gag ctg cgg gtg cgg ctg gag gag cgg cgc	2803
Ala Asp Trp Val Tyr Asn Glu Leu Arg Val Arg Leu Glu Glu Arg Arg	
885 890 895	
ggt cgc cga gcc cta cgc ttg tgt ctg gag gac cga gat tgg ctg cct	2851
Gly Arg Arg Ala Leu Arg Leu Cys Leu Glu Asp Arg Asp Trp Leu Pro	
900 905 910 915	
ggc cag acg ctc ttc gag aac ctc tgg gct tcc atc tat ggg agc cgc	2899
Gly Gln Thr Leu Phe Glu Asn Leu Trp Ala Ser Ile Tyr Gly Ser Arg	
920 925 930	
aag act cta ttt gtg ctg gcc cac acg gac cgc gtc agt ggc ctc ctg	2947
Lys Thr Leu Phe Val Leu Ala His Thr Asp Arg Val Ser Gly Leu Leu	
935 940 945	
cgc acc agc ttc ctg ctg gct cag cag cgc ctg ttg gaa gac cgc aag	2995
Arg Thr Ser Phe Leu Leu Ala Gln Gln Arg Leu Leu Glu Asp Arg Lys	

950	955	960	
gac gtg gtg gtg ttg gtg atc ctg cgt ccg gat gcc cac cgc tcc cgc 3043			
Asp Val Val Val Leu Val Ile Leu Arg Pro Asp Ala His Arg Ser Arg			
965	970	975	
tat gtg cga ctg cgc cag cgt ctc tgc cgc cag agt gtg ctc ttc tgg 3091			
Tyr Val Arg Leu Arg Gln Arg Leu Cys Arg Gln Ser Val Leu Phe Trp			
980	985	990	995
ccc cag cag ccc aac ggg cag ggg ggc ttc tgg gcc cag ctg agt aca 3139			
Pro Gln Gln Pro Asn Gly Gln Gly Gly Phe Trp Ala Gln Leu Ser Thr			
	1000	1005	1010
gcc ctg act agg gac aac cgc cac ttc tat aac cag aac ttc tgc cgg 3187			
Ala Leu Thr Arg Asp Asn Arg His Phe Tyr Asn Gln Asn Phe Cys Arg			
	1015	1020	1025
gga cct aca gca gaa tag ctgagagcaa cagctggaaa cagctgcac 3235			
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	1030		
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agggtagttt tcttcccatg catctttcag gagagtgaag atagacacca gaccacaca 3415			
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<210> 4

<211> 1032

<212> PRT

<213> Mus musculus

<400> 4

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Ala Ala Val Leu Ala Glu Thr Leu Ala Leu Gly Thr Leu Pro Ala Phe  
20 25 30  
Leu Pro Cys Glu Leu Lys Pro His Gly Leu Val Asp Cys Asn Trp Leu  
35 40 45  
Phe Leu Lys Ser Val Pro Arg Phe Ser Ala Ala Ala Ser Cys Ser Asn  
50 55 60  
Ile Thr Arg Leu Ser Leu Ile Ser Asn Arg Ile His His Leu His Asn  
65 70 75 80  
Ser Asp Phe Val His Leu Ser Asn Leu Arg Gln Leu Asn Leu Lys Trp  
85 90 95  
Asn Cys Pro Pro Thr Gly Leu Ser Pro Leu His Phe Ser Cys His Met  
100 105 110  
Thr Ile Glu Pro Arg Thr Phe Leu Ala Met Arg Thr Leu Glu Glu Leu  
115 120 125  
Asn Leu Ser Tyr Asn Gly Ile Thr Thr Val Pro Arg Leu Pro Ser Ser  
130 135 140  
Leu Val Asn Leu Ser Leu Ser His Thr Asn Ile Leu Val Leu Asp Ala  
145 150 155 160  
Asn Ser Leu Ala Gly Leu Tyr Ser Leu Arg Val Leu Phe Met Asp Gly  
165 170 175  
Asn Cys Tyr Tyr Lys Asn Pro Cys Thr Gly Ala Val Lys Val Thr Pro

	180		185		190
Gly	Ala	Leu	Leu	Gly	Leu
		Ser	Asn	Leu	Thr
				His	Leu
				Ser	Val
				Lys	Tyr
	195		200		205
Asn	Asn	Leu	Thr	Lys	Val
		Pro	Arg	Gln	Leu
				Pro	Pro
				Ser	Leu
				Glu	Tyr
	210		215		220
Leu	Leu	Val	Ser	Tyr	Asn
					Leu
					Ile
					Val
					Lys
					Leu
					Gly
					Pro
					Glu
					Asp
					Leu
225			230		235
240					
Ala	Asn	Leu	Thr	Ser	Leu
					Arg
					Val
					Leu
					Asp
					Val
					Gly
					Gly
					Asn
					Cys
					Arg
	245		250		255
Arg	Cys	Asp	His	Ala	Pro
					Asn
					Pro
					Cys
					Ile
					Glu
					Cys
					Gly
					Gln
					Lys
					Ser
	260		265		270
Leu	His	Leu	His	Pro	Glu
					Thr
					Phe
					His
					His
					Leu
					Ser
					His
					Leu
					Glu
					Gly
	275		280		285
Leu	Val	Leu	Lys	Asp	Ser
					Ser
					Leu
					His
					Thr
					Leu
					Asn
					Ser
					Ser
					Trp
					Phe
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Gln	Gly	Leu	Val	Asn	Leu
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					Val
					Leu
					Asp
					Leu
					Ser
					Glu
					Asn
					Phe
					Leu
305			310		315
320					
Tyr	Glu	Ser	Ile	Asn	His
					Thr
					Asn
					Ala
					Phe
					Gln
					Asn
					Leu
					Thr
					Arg
					Leu
	325		330		335
Arg	Lys	Leu	Asn	Leu	Ser
					Phe
					Asn
					Tyr
					Arg
					Lys
					Lys
					Val
					Ser
					Phe
					Ala
	340		345		350
Arg	Leu	His	Leu	Ala	Ser
					Ser
					Phe
					Lys
					Asn
					Leu
					Val
					Ser
					Leu
					Gln
					Glu
	355		360		365
Leu	Asn	Met	Asn	Gly	Ile
					Phe
					Phe
					Arg
					Ser
					Leu
					Asn
					Lys
					Tyr
					Thr
					Leu
	370		375		380
Arg	Trp	Leu	Ala	Asp	Leu
					Pro
					Lys
					Leu
					His
					Thr
					Leu
					His
					Leu
					Gln
					Met
385			390		395
400					
Asn	Phe	Ile	Asn	Gln	Ala
					Gln
					Leu
					Ser
					Ile
					Phe
					Gly
					Thr
					Phe
					Arg
					Ala
	405		410		415
Leu	Arg	Phe	Val	Asp	Leu
					Ser
					Asp
					Asn
					Arg
					Ile
					Ser
					Gly
					Pro
					Ser
					Thr

420	425	430
Leu Ser Glu Ala Thr Pro Glu Glu Ala Asp Asp Ala Glu Gln Glu Glu		
435	440	445
Leu Leu Ser Ala Asp Pro His Pro Ala Pro Leu Ser Thr Pro Ala Ser		
450	455	460
Lys Asn Phe Met Asp Arg Cys Lys Asn Phe Lys Phe Thr Met Asp Leu		
465	470	475
Ser Arg Asn Asn Leu Val Thr Ile Lys Pro Glu Met Phe Val Asn Leu		
485	490	495
Ser Arg Leu Gln Cys Leu Ser Leu Ser His Asn Ser Ile Ala Gln Ala		
500	505	510
Val Asn Gly Ser Gln Phe Leu Pro Leu Thr Asn Leu Gln Val Leu Asp		
515	520	525
Leu Ser His Asn Lys Leu Asp Leu Tyr His Trp Lys Ser Phe Ser Glu		
530	535	540
Leu Pro Gln Leu Gln Ala Leu Asp Leu Ser Tyr Asn Ser Gln Pro Phe		
545	550	555
Ser Met Lys Gly Ile Gly His Asn Phe Ser Phe Val Ala His Leu Ser		
565	570	575
Met Leu His Ser Leu Ser Leu Ala His Asn Asp Ile His Thr Arg Val		
580	585	590
Ser Ser His Leu Asn Ser Asn Ser Val Arg Phe Leu Asp Phe Ser Gly		
595	600	605
Asn Gly Met Gly Arg Met Trp Asp Glu Gly Gly Leu Tyr Leu His Phe		
610	615	620
Phe Gln Gly Leu Ser Gly Leu Leu Lys Leu Asp Leu Ser Gln Asn Asn		
625	630	635
Leu His Ile Leu Arg Pro Gln Asn Leu Asp Asn Leu Pro Lys Ser Leu		
645	650	655
Lys Leu Leu Ser Leu Arg Asp Asn Tyr Leu Ser Phe Phe Asn Trp Thr		

660	665	670
Ser Leu Ser Phe Leu Pro Asn Leu Glu Val Leu Asp Leu Ala Gly Asn		
675	680	685
Gln Leu Lys Ala Leu Thr Asn Gly Thr Leu Pro Asn Gly Thr Leu Leu		
690	695	700
Gln Lys Leu Asp Val Ser Ser Asn Ser Ile Val Ser Val Val Pro Ala		
705	710	715
Phe Phe Ala Leu Ala Val Glu Leu Lys Glu Val Asn Leu Ser His Asn		
725	730	735
Ile Leu Lys Thr Val Asp Arg Ser Trp Phe Gly Pro Ile Val Met Asn		
740	745	750
Leu Thr Val Leu Asp Val Arg Ser Asn Pro Leu His Cys Ala Cys Gly		
755	760	765
Ala Ala Phe Val Asp Leu Leu Leu Glu Val Gln Thr Lys Val Pro Gly		
770	775	780
Leu Ala Asn Gly Val Lys Cys Gly Ser Pro Gly Gln Leu Gln Gly Arg		
785	790	795
Ser Ile Phe Ala Gln Asp Leu Arg Leu Cys Leu Asp Glu Val Leu Ser		
805	810	815
Trp Asp Cys Phe Gly Leu Ser Leu Leu Ala Val Ala Val Gly Met Val		
820	825	830
Val Pro Ile Leu His His Leu Cys Gly Trp Asp Val Trp Tyr Cys Phe		
835	840	845
His Leu Cys Leu Ala Trp Leu Pro Leu Leu Ala Arg Ser Arg Arg Ser		
850	855	860
Ala Gln Ala Leu Pro Tyr Asp Ala Phe Val Val Phe Asp Lys Ala Gln		
865	870	875
Ser Ala Val Ala Asp Trp Val Tyr Asn Glu Leu Arg Val Arg Leu Glu		
885	890	895
Glu Arg Arg Gly Arg Arg Ala Leu Arg Leu Cys Leu Glu Asp Arg Asp		



**【Brief Description of the Drawings】**

**【Fig. 1】** FIG. 1 shows a gene maps of TLR9 knockout mice in the present invention and wild-type mice.

**【Fig. 2】** FIG. 2 shows the result of Southern blot analysis of TLR9 knockout mice in the present invention.

**【Fig. 3】** FIG. 3 shows the result of Northern blot analysis of spleen cells from TLR9 knockout mice in the present invention.

**【Fig. 4】** FIG. 4 shows the result of comparing the sequence of amino acids from TLR9 knockout mice in the present invention and the sequence of amino acids from wild-type mice.

**【Fig. 5】** FIG. 5 shows the result of measurement of  $\text{TNF}\alpha$ , IL-6 or IL-12 production induced by CpG ODN, PGN or LPS in TLR9 knockout mice in the present invention and in wild-type mice.

**【Fig. 6】** FIG. 6 shows the result of cellular proliferation response induced by CpG ODN or LPS in TLR9 knockout mice in the



present invention or in wild-type mice.

【Fig. 7】 FIG. 7 shows the result of measurement of IL-12 production induced by CpG ODN or LPS in TLR9 knockout mice in the present invention or in wild-type mice.

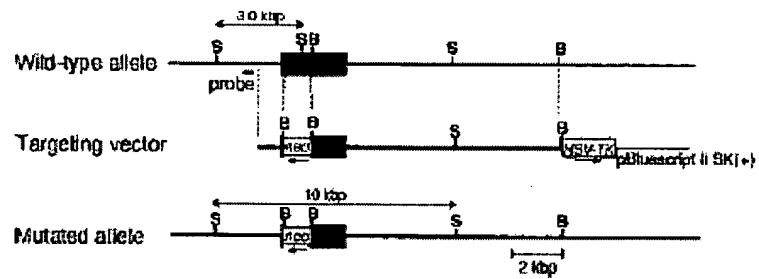
【Fig. 8】 FIG. 8 shows the result of expression of CD40, CD80, CD86, and MHC class II induced by CpG ODN or LPS in TLR9 knockout mice in the present invention and in wild-type mice.

【Fig. 9】 FIG. 9 shows the result of activation of NF- $\kappa$ B induced by CpG ODN or LPS in TLR9 knockout mice in the present invention or in wild-type mice.

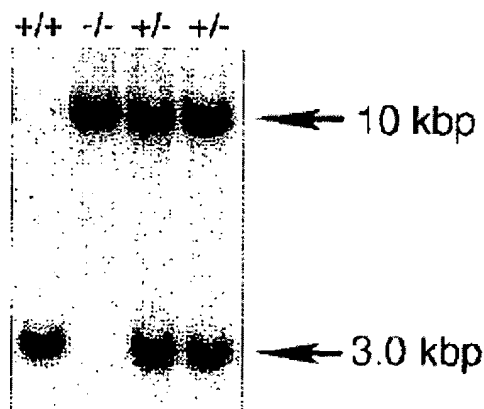
【Fig. 10】 FIG. 10 shows the result of activation of JNK induced by CpG ODN or LPS in TLR9 knockout mice in the present invention or in wild-type mice.

【Fig. 11】 FIG. 11 shows the result of activation of IRAK induced by CpG ODN or LPS in TLR9 knockout mice in the present invention or in wild-type mice.

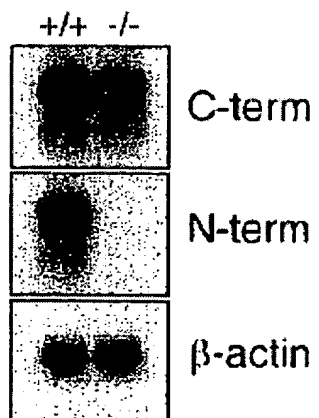
【Fig. 1】



【Fig. 2】



【Fig. 3】



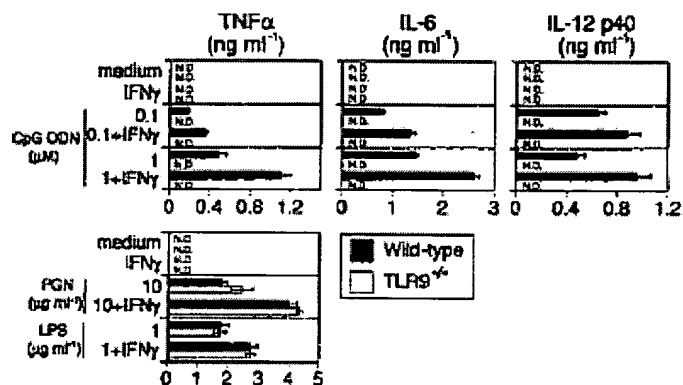
【Fig. 4】

	87		90				96		100									110						
+/+	TCC	AAC	CTG	CGG	CAG	CTG	AAC	CTC	AAG	TGG	AAC	TGT	CCA	CCC	ACT	GGC	CTT	AGC	CCC	TTG	CAC	TTC	TCT	TGC
	S	N	L	R	Q	L	N	L	K	W	N	C	P	P	T	G	L	S	P	L	H	F	S	C
-/-	S	N	L	R	Q	L	N	L	K	W	I	L	S	T	C	P	R	R	I	R	I	N	D	P
	87			90						96														

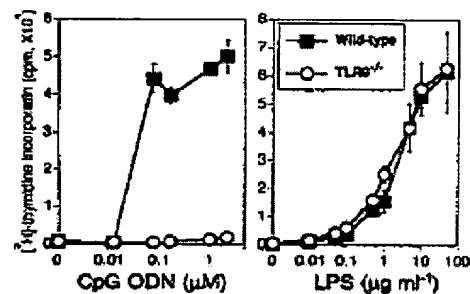
  

									120									130						
+/+	CAC	ATG	ACC	ATT	GAG	CCC	AGA	ACC	TTC	CTG	GCT	ATG	CGT	ACA	CTG	GAG	GAG	CTG	AAC	CTG	AGC	TAT	AAT	GGT
	H	M	T	I	E	P	R	T	F	L	A	M	R	T	L	E	E	L	N	L	S	Y	N	G
-/-	T	P	V	R	F	I	L	S	F	Y	C	R	S	P	Q	K	N	S	S	R	R	R	*	
	ACA	CCC	GTG	CGT	TTC	ATT	CTG	TCT	TTT	TAT	TGC	CGA	TCC	CCT	CAG	AAG	AAC	TCG	TCA	AGA	AGG	CGA	TAG	

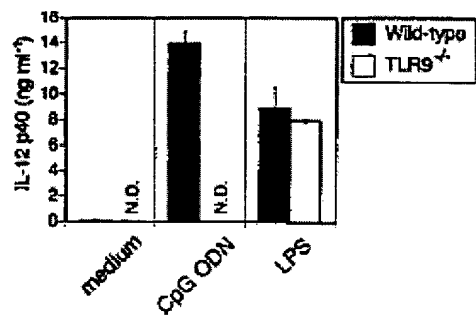
【Fig. 5】



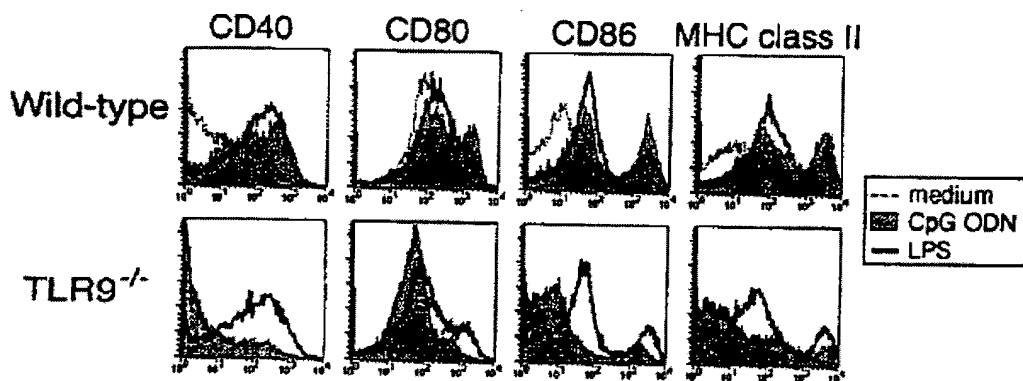
【Fig. 6】



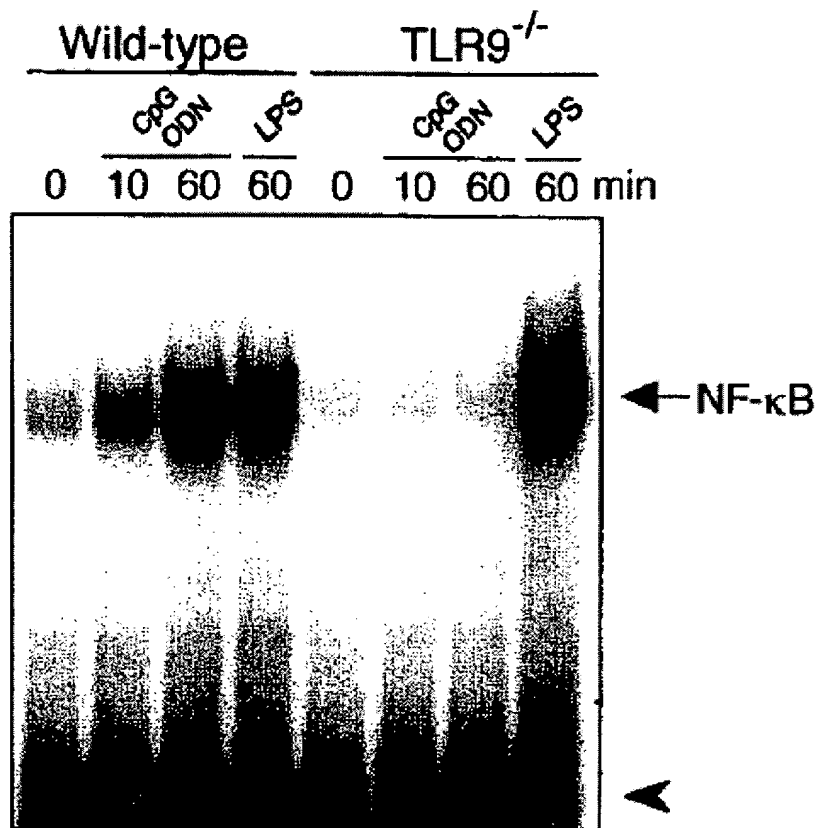
【Fig. 7】



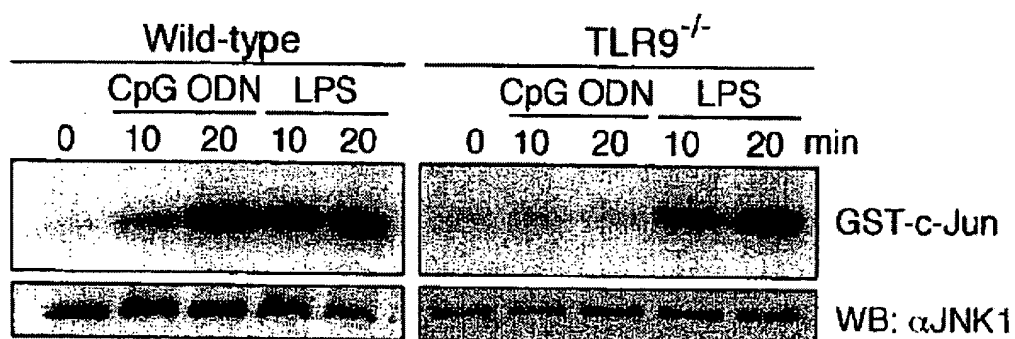
【Fig. 8】



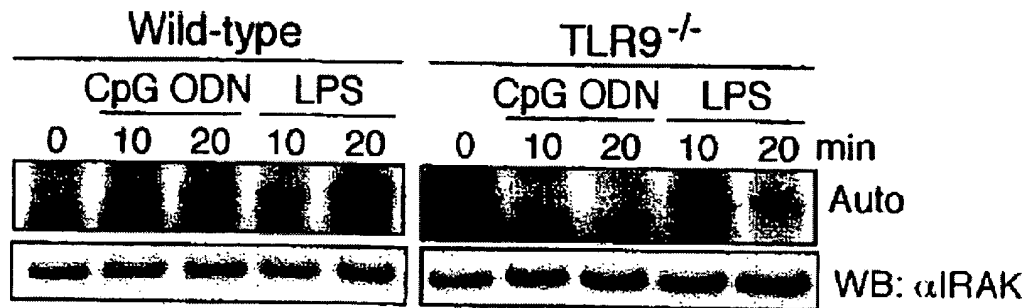
【Fig. 9】



【Fig. 10】



[Fig. 11]



【Name of Document】 Abstract

【Abstract】

【The Object】 The present invention provides a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence, a genomic DNA encoding it, an experimental animal model useful for examining responsiveness of a host immune cell against a bacterial infectious disease.

【Means to Solve the Problem】 DNA encoding a receptor protein specifically recognizing bacterial DNA having an unmethylated CpG sequence is screened by BLAST search, a number of EST clones having high homology with various TLRs is screened, these clones are used as a probe to isolate a full-length cDNA from mouse macrophage cDNA library, and the sequence of bases of the cDNA is analyzed to confirm that it is TLR9 comprising a conserved regions such as LRR and TIR regions, and then a knockout mouse is produced to confirm that TLR9 is a receptor protein of oligonucleotides having an unmethylated CpG sequence of bacterial DNA.